EFFECT OF DIETARY PROTEIN SOURCE ON SATIETY, POSTPRANDIAL BLOOD BIOMARKERS, AND METABOLISM

BY

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DISSERTATION

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Abstract

High protein diets have become an increasingly popular dieting strategy, but the extent to which different protein sources are similarly anorexigenic and the mechanisms involved are less certain. It was previously observed that male Sprague-Dawley rats given a 35% egg white protein meal at the first meal of the day were more satiated in the initial hours following the meal than when provided a 35% wheat gluten protein meal. The objective of this study was to examine the effect of the source and level of protein on subsequent feeding behavior, metabolism, and changes in plasma amino acids and insulin levels in order to identify possible mechanisms involved in differences of satiety. Rats were entrained to a meal-feeding schedule, which included an overnight fast before being provided a 30-minute treatment meal. The treatment meals consisted of one of four isocaloric diets equivalent to 10-20% of average daily intake and were administered one hour into the dark phase: 20% egg white protein (20EW), 20% wheat gluten protein (20WG), 35% egg white protein (35EW), or 35% wheat gluten protein (35WG). Ad libitum access to a control diet was made available later in the dark phase. Blood plasma was collected from rats surgically implanted with jugular catheters at baseline and at 30-minute intervals for two hours following test meal ingestion, and analyzed for amino acid and insulin concentrations. Separate cohorts of rats were assessed for feeding behavior and metabolism following acute and chronic treatment paradigms. Egg white meals increased total amino acids, as well as specific amino acids including lysine, isoleucine, valine, and tryptophan more than wheat gluten meals ($P<0.005$). Insulin levels reflected level of protein rather than source. Rats fed egg white displayed decreased food intake at the subsequent meal compared to wheat gluten, regardless of protein level or sex (both $P<0.005$). The respiratory exchange ratio following ingestion of 35EW was lower than the other treatment meals for several hours

following ingestion ($P<0.001$), however, energy expenditure did not differ among treatments groups. When administered over the course of 30 days, the treatments had no effect on changes in body weight, body composition, energy intake, or energy expenditure. Results confirmed that meals containing egg white protein induced greater satiety than wheat gluten protein, which corresponded to increased postprandial plasma amino acids and lowered respiratory exchange ratio following egg white protein meals. Although dietary source of protein has significant short term implications for satiety and metabolism, manipulation of the protein component of a single meal of the day has minimal long term effects on body weight and composition. Altogether, these results emphasize the importance of considering protein source when designing diets to control appetite.

Dedicated in loving memories of my grandma,

Ngoại (1929 – 2017)

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Chapter 1: Introduction

In recent decades, there has been an increase in the incidence of obesity and metabolic syndrome due to excess weight gain, leading many health professionals to extensively investigate solutions to this problem. Following a low calorie diet by limiting food consumption is difficult for many to maintain (Ayyad & Andersen, 2000), so it is of huge interest to understand how we can choose foods that maximize satiety to support weight loss (Drapeau et al., 2007; Soenen & Westerterp-Plantenga, 2008). Among macronutrients, that is carbohydrates, fats, and protein, protein has been shown to have the greatest satiety effect (Bensaid et al., 2002; Li & Anderson, 1982; Rolls, Hetherington, & Burley, 1988; Westerterp-Plantenga, Nieuwenhuizen, Tomé, Soenen, & Westerterp, 2009). Further evidence suggests that protein-induced satiety may be dependent on where the dietary protein is coming from, referred to here as protein source (Abou-Samra, Keersmaekers, Brienza, Mukherjee, & Macé, 2011; Bayham, Greenway, Johnson, & Dhurandhar, 2014; Bendtsen, Lorenzen, Bendsen, Rasmussen, & Astrup, 2013). With weight gain an ever increasing issue, weight loss strategy has shifted away from low fat diets in favor of high protein diets (Jeor et al., 2001). Many versions of these diets encourages high protein intake in order to lose weight with promises of minimal hunger (Astrup, Larsen, & Harper, 2004). The increased popularity of such diets as a strategy to losing weight and maintaining weight loss makes this a critical time for better understanding of protein-induced satiety.

High-protein meals have been shown to reduce subsequent food intake in both rodent (Bensaid et al., 2002; Li & Anderson, 1982) and human subjects (Rolls et al., 1988; Stubbs, Van Wyk, Johnstone, & Harbron, 1996) compared to isocaloric meals from carbohydrate or fat. A recent review of twenty-four studies that compared the acute satiety effects of meals at various

protein levels found that higher protein meals had a positive satiety effect (i.e. decreased perceived hunger and increased satiety, increased satiety hormones, lower subsequent intake) in 17 studies (Heather J Leidy et al., 2015). Beneficial satiety effects of high protein meals were observed in both lean and overweight or obese subjects. This positive effect of high protein meals increasing satiety appears to be limited to meals providing protein in the form of solid food and not beverage meals, and in a threshold quantity. All of the studies that resulted in positive findings provided protein preload meals in the form of a solid meal. The three studies using beverage preload had null results further supporting the literature that energy in beverage form generally produces a weaker satiety response (Heather J Leidy, Apolzan, Mattes, & Campbell, 2010; Mourao, Bressan, Campbell, & Mattes, 2007). Furthermore, there appears to be a minimum amount of protein ingestion required to stimulate satiety response. Early evidence suggests a high protein meal containing a minimum threshold amount of 25-30 grams of protein needs to be consumed within a meal to elicit a satiety response (Paddon-Jones & Leidy, 2014). Overall, ingestion of dietary protein in solid food and in a large enough quantity increases satiety and decreases subsequent meal size.

Beyond the acute satiety effects of high protein meals, high protein diets also have positive effects on body weight. Overweight and obese subjects on high protein diets (25-35% energy from protein, 1.07-1.60 gram protein per kg per day) for at least 4 weeks lose more body weight and fat mass than patients consuming a standard protein diet (12-18% energy from protein, 0.55-0.88 gram protein per kg per day) even though both groups were prescribed equally low fat, energy restricted diets (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012). Furthermore, increased protein can aid in long-term weight maintenance. The Diogenes Study is one of the largest weight-loss maintenance studies completed spanning eight centers and over

700 subjects (Larsen et al., 2010). Overweight and obese individuals went through an 8-week energy restricted diet to lose at least 8% of their body weight, prior to being randomized into a high protein (25% energy from protein) or low protein (13% energy from protein) diet group for six months. The individuals in the high protein diet group regained less weight and were more likely to complete the study, suggesting that the increased dietary protein makes it easier to adhere to dietary goals (Larsen et al., 2010). In general, data suggests adherence to a high protein diet of at least 1.2 grams of protein per kg per day is a beneficial method for controlling appetite to in turn support weight loss and weight maintenance (Heather J Leidy et al., 2015). Increasing overall protein consumption is an advantageous method for losing weight in overweight and obese individuals, as well as maintaining weight loss.

The recommendation of >25% energy from protein or 1.2 grams of protein per kg per day is greater than the current recommended dietary allowance (RDA) of protein of 46 grams per day for adult women and 56 grams per day for adult men (Micronutrients, 2005). Most Americans are consuming enough protein to meet RDA, with protein making up roughly 15% of total energy intake and less than 10% of the population consuming inadequate levels of protein (Fulgoni, 2008). Although the RDA of protein is made to be adequate for normal physiological processes, these appear to not be high enough to promote satiety, weight management, and various other health factors. Further, there is increasing evidence that protein intake above the levels recommended in the Dietary Guidelines may have beneficial effects beyond controlling body weight. These benefits include muscle mass preservation during aging and illness, controlling glycemia, improving cardiometabolic measures, and optimizing protein synthesis (Arentson-Lantz, Clairmont, Paddon-Jones, Tremblay, & Elango, 2015; Donald K Layman et al., 2015). The high levels of protein suggested for satiety and weight management still fall within

the acceptable macronutrient distribution range (AMDR) that 10-35% energy intake should come from protein. This suggests a major difference between minimum and optimal levels of dietary protein consumption, and many groups of the population could benefit from increased protein intake.

Protein sources each have unique properties which differentially impact human and non-human animal health that can be described by protein quality. Protein quality describes the evaluation of dietary protein sources for its ability to provide amino acids to match metabolic demands (Lee et al., 2016; Millward, Layman, Tomé, & Schaafsma, 2008). This largely depends on the essential amino acid profile that makes up the protein source, but also the many factors that alter bioavailability of those amino acids. Bioavailability can be modulated by a number of factors which includes digestibility, amino acid ratios, rate of passage through the gastrointestinal tract, and anti-nutritional factors (such as fiber and trypsin inhibitors) (Gilani, Xiao, & Cockell, 2012). Although protein quality had previously been evaluated by its ability to maximize growth of young rats (protein efficiency ratio, PER), the current method for assessing protein quality is the protein digestibility-corrected amino acid score (PDCAAS) (Gertjan Schaafsma, 2005). PDCAAS assesses proteins based off of the first limiting essential amino acid and corrects for its digestibility. However, there has been considerable development in understanding the role of protein on health in the last three decades since the introduction of PDCAAS which extends beyond supporting growth and maintenance, and there are many limitations to this method of protein evaluation. While assessment of protein quality does not directly consider its satiety effects, a protein's digestive properties and amino acid bioavailability will likely affect satiety signaling mechanisms by increasing postprandial amino acids availability (Bendtsen et al., 2013).

In addition to the amount of protein within a meal and in the overall diet, there also needs to be consideration of whether all protein sources have the same effects on satiety. Because the various protein sources differ in amino acid composition and food matrix, they can vary in their ability to alter physiological functions, including those involved in satiety. Many studies comparing the effect of protein source on satiety have focused on the two milk proteins, whey and casein. A relatively recent review of 31 studies comparing the two milk protein sources found whey to be associated with greater satiety 1-2 hours following a test meal, whereas casein having greater satiety 4-6 hours after the test meal (Bendtsen et al., 2013). Results from studies measuring plasma amino acid appearance in the blood following consumption of these two protein sources suggests whey is digested more rapidly with plasma amino acids appearing more quickly and lasting more briefly compared to changes in plasma amino acid following casein consumption (Boirie et al., 1997; Hall, Millward, Long, & Morgan, 2003). Unlike whey, the acidic environment of the stomach causes casein to form clots, slowing down gastric emptying and amino acid absorption (Bendtsen et al., 2013; Boirie et al., 1997). Slower gastric emptying prolongs stomach distention to increase satiety (Clegg & Shafat, 2010). This comparison between casein and whey is just an example of how the digestion and absorption of different protein sources may result in varied satiety responses. A more extensive comparison of studies assessing the satiety effect of different protein sources is provided in Table 1. This table demonstrates that there are differences in protein-induced satiety among protein sources. However, the mechanisms underlying the unique satiety responses from different protein sources is not well understood.

1.1 Mechanisms underlying protein-induced satiety

While it is uncertain what the underlying mechanism involved in differential satiety responses from various protein sources, several mechanisms which have been proposed to be involved in protein-induced satiety including amino acid availability, gluconeogenesis, satiety hormones, and diet-induced thermogenesis.

1.1.1 Amino acid availability

The most direct explanation for protein induced satiety is the increase in amino acids from protein ingestion. Evidence for an increase in overall amino acid availability inducing satiety has been around for 60 years (Mellinkoff, Frankland, Boyle, & Greipel, 1956). A 1956 study demonstrated dietary consumption of protein and amino acids to increase serum amino acid, correlated with decreased appetite. This relationship between serum amino acid and satiety became known as the aminostatic hypothesis. It is suggested that plasma amino acids beyond that which is required for protein synthesis serves as a satiety signal causing decreases in food intake. Numerous other studies since the publication of the aminostatic hypothesis have also shown postprandial plasma amino acids are correlated with increased satiety (Boirie et al., 1997; Hall et al., 2003; M. A. Veldhorst et al., 2009b; M. A. B. Veldhorst, A. G. Nieuwenhuizen, A. Hochstenbach-Waelen, K. R. Westerterp, et al., 2009). Differences in increases plasma amino acid concentrations following ingestion of protein from varying sources can uniquely influence satiety.

In addition to total concentration of amino acids in the blood, individual amino acids have been thought to play a specific role in signaling satiety. Tryptophan is a precursor to the anorexigenic neurotransmitter serotonin, known to decrease appetite and food intake (Fernstrom, 1985; Shor-Posner, Grinker, Marinescu, Brown, & Leibowitz, 1986). Increases in the ratio of

tryptophan to large neutral amino acids by dietary protein ingestion may potentially support brain uptake of tryptophan, serotonin synthesis and therefore suppression of appetite (Fernstrom, 1985). However, whether dietary proteins with high levels of tryptophan or added tryptophan can suppress hunger is questionable (Nieuwenhuizen et al., 2009; M. A. Veldhorst et al., 2009a). Leucine is also thought to have a specific function as a satiety signaling amino acid. Leucine is known to stimulate protein synthesis by activating mammalian target of rapamycin (mTOR) in skeletal muscle (Garlick, 2005). It is suggested that leucine signals availability of amino acids and be involved in intracellular mechanism of detecting the quantity and/or quality of dietary protein (Garlick, 2005; D. K. Layman & Baum, 2004). Injection of leucine directly into the third ventricle of the brain increases hypothalamic mTOR signaling and decreases food intake and body weight (Cota et al., 2006). Mice given free access to a high-fat diet and supplementation of dietary leucine gained less weight, as well as were protected from development of hyperglycemia and hypercholesterolemia (Zhang et al., 2007). A study in which rats received isomolar gavages of individual amino acids revealed arginine, lysine, and glutamate to have the greatest decrease in food intake during the first hour after administration compared to the other 17 amino acids (Jordi et al., 2013). All three of these amino acids slowed gastric emptying and neuronal activity, but were found to do so through varying mechanisms. Whereas arginine and glutamate relied on the area postrema for its anoretic effects, lysine appeared dependent on intact vagal afferent nerves. While it does appear that individual amino acids have specific satiety signaling roles, further investigation is necessary to identify which amino acids effect satiety, and the extent to which dietary sources composed of different amino acids have differential effects on satiety.

1.1.2 Gluconeogenesis

Amino acids from dietary protein can be fed through gluconeogenesis to modulate glucose homeostasis and thereby contribute to satiety. In the fed state, gluconeogenesis primarily occurs in the liver. Rats fed a high protein diet had increased hepatocyte expression of phosphoenolpyruvate carboxykinase (PEPCK), a rate limiting enzyme of gluconeogenesis (Azzout-Marniche et al., 2007). Increased gluconeogenesis along with decreased appetite has also been demonstrated in human subjects fed a high protein diet (Veldhorst, Westerterp, & Westerterp-Plantenga, 2012). Furthermore, decreases in blood glucose has been shown to be associated with meal initiation (Melanson, Westerterp-Plantenga, Saris, Smith, & Campfield, 1999). Beyond hepatic gluconeogenesis, it has been suggested that intestinal gluconeogenesis leading to portal sensing of glucose through portal vagus afferent fibers contribute to protein-induced satiety (Mithieux et al., 2005; Penhoat et al., 2011). Validity of these claims have yet to be proven and interpretation of data from these studies remain controversial (Previs, Brunengraber, & Brunengraber, 2009; Watford, 2005). Overall, amino acids in excess of that required for protein synthesis can produce glucose through gluconeogenesis, and thereby contribute to protein-induced satiety by preventing decreases of glucose and signaling energy availability to the brain.

Different protein sources vary in their composition of amino acids that can be used by gluconeogenesis. Of the twenty amino acids, leucine and lysine are exclusively ketogenic amino acids, converted into ketone bodies through ketogenesis (Harvey & Ferrier, 2011). If protein-induced gluconeogenesis contributes to the satiety effect, then proteins with more gluconeogenic amino acids may be more likely to increase satiety or increase satiety to a greater extent.

1.1.3 Satiety signaling hormones

It is suggested that during protein digestion, amino acids and oligopeptides are detected by chemoreceptors on the lumen of the small intestine triggering the release of various satiety inducing hormones from enteroendocrine cells (Raybould et al., 2006). These gut satiety hormones include cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and peptide YY (PYY), and can transmit information about energy intake by interacting with vagal nerve afferents. The vagal afferent nerve synapses in the nucleus of the solitary tract (NTS) in the brainstem which then transmits information to the hypothalamus. Release of CCK from enterocytes is highly responsive to presence of protein and long chain fatty acids, causing rapid increases of CCK during food ingestion to function as a within meal satiety signal (T. H. Moran & Dailey, 2011). Amino acids and oligopeptide detection by the intestinal epithelium triggers the release of CCK. Additionally, CCK secretion has a role in slowing down gastric emptying and stimulating pancreatic and gall bladder secretion to aid in the digestive process (Raybould, 2007). CCK is secreted in amounts proportional to ratings of satiety (Burton-Freeman, 2008). GLP-1 and PYY are described as between-meal satiety signals since they increase more slowly than CCK in response to food intake and remain elevated for several hours following a meal (T. Moran, 2009). GLP-1 is most responsive to carbohydrate ingestion, but there is evidence to support its response to proteins and amino acids as well. GLP-1 release has been shown to occur in response to leucine, isoleucine, and dairy protein administration in vitro (Chen & Reimer, 2009). GLP-1 concentrations were higher following a 30% protein meal than a 10% protein meal, corresponding with increased satiety (Lejeune, Westerterp, Adam, Luscombe-Marsh, & Westerterp-Plantenga, 2006). Administration of PYY reduces food intake and weight gain in rats, and in humans decreases appetite and food intake (Rachel L Batterham et al., 2002; R. L. Batterham et al., 2006). High protein meals produced a greater increase in plasma PYY levels in

both lean and obese subjects (R. L. Batterham et al., 2006). Acting in opposition to previously mentioned satiety hormones is the hunger hormone, ghrelin. Ghrelin is released from the stomach and its release inhibited by glucose and amino acids presence in the gastrointestinal tract (Overduin, Frayo, Grill, Kaplan, & Cummings, 2005). Levels of ghrelin peak just before a meal and decrease after food ingestion (Cummings et al., 2001). Further, a high protein breakfast decreased postprandial ghrelin secretion to a greater extent than a high carbohydrate breakfast (Blom et al., 2006). In summary, protein ingestion alters hormone release in the gastrointestinal tract that function as feedback signals to regulate food intake.

Insulin is most notable for its role in controlling glucose homeostasis, with its release from pancreatic islet β-cells triggered by increased blood glucose. Insulin is also considered to decrease appetite by acting as an adiposity negative feedback signal, along with leptin (Morton, Cummings, Baskin, Barsh, & Schwartz, 2006; Williams & Elmquist, 2012). The amount of plasma insulin (and leptin) correlates with amount of body fat (Considine et al., 1996). Insulin signaling in the brain acts as a negative feedback signal to regulate body fat by increasing sensitivity to satiety signals from food ingestion and thereby reducing food intake (Woods, Lutz, Geary, & Langhans, 2006). Administration of insulin directly to the brain enhances the ability of CCK to reduce meal size (Figlewicz, Stein, West, Porte, & Woods, 1986; Riedy, Chavez, Figlewicz, & Woods, 1995). It is suggested that insulin can interact with glucose in the liver to have an acute satiety effect, reducing food intake and stimulating meal termination, and this process requires intact vagus nerves (Woods et al., 2006). Postprandial increases in plasma insulin correspond with increased satiety and decreased food intake at the following meal (Holt, Brand Miller, & Petocz, 1996; Verdich et al., 2001). While exclusive ingestion of protein is a weak stimulus for insulin release, the combine intake of protein and carbohydrate synergistically

increases plasma insulin concentrations (Nuttall, Gannon, Wald, & Ahmed, 1985; Nuttall, Mooradian, Gannon, Billington, & Krezowski, 1984; Pallotta & Kennedy, 1968; Rabinowitz, Merimee, Maffezzoli, & Burgess, 1966). A combination of arginine, phenylalanine, and leucine with glucose has been shown to produce a greater increase in plasma insulin compared to arginine with glucose (van Loon, Saris, Verhagen, & Wagenmakers, 2000). Postprandial changes in plasma insulin corresponded closely to changes in plasma leucine, phenylalanine, and tyrosine (van Loon et al., 2000). Overall, protein and amino acid stimulates insulin release to in turn influence satiety, a process that requires carbohydrate ingestion.

1.1.4 Diet-induced thermogenesis

Increases in energy expenditure following protein consumption has been suggested to be involved in protein-induced satiety. Both physical activity and food ingestion can increase energy expenditure above basal metabolic rates (Westerterp, 2004). While the amount of energy provided by a meal is the largest driving factor of diet-induced thermogenesis, ingestion of dietary protein is also a determinant of increases in energy expenditure in the hours following meal consumption (Halton & Hu, 2004; Westerterp, 2004). High protein meals (68% of energy from protein) produce greater diet-induced thermogenesis compared to lower protein meals, correlating with increased satiety (Crovetti, Porrini, Santangelo, & Testolin, 1998). All 15 studies included in a review of randomized controlled trials investigating the effects of high protein diets (14-100% of energy from protein) on thermogenesis found high protein diets to increase thermogenesis to a greater extent than control lower protein diets (Halton & Hu, 2004). The increase in energy expenditure is proposed to be due to the body lacking the capacity to store excess protein and in turn has to quickly metabolize excess protein intake (Halton & Hu, 2004). Postprandial protein metabolism is higher; energy expenditure for ATP synthesis from

amino acids is 99.2 and 153.2 kJ/ATP, whereas glucose is 91.0 kJ/ATP (Van Milgen, 2002). It is also suggested that increased oxygen consumption and body temperature due to increased energy expenditure caused feelings of oxygen deprivation inducing satiety (Westerterp-Plantenga, Rolland, Wilson, & Westerterp, 1999). Further, there is some evidence that different sources of protein can influence the thermogenesis that is produced. A diet of 29% energy from pork protein was demonstrated to elevate 24 hour energy expenditure to a greater extent than a diet of the same amount of protein coming from soy protein (Mikkelsen, Toubro, & Astrup, 2000). Individual amino acids vary in the structure of their carbon side chains, which have unique metabolic fates and different energetic costs during catabolism (Van Milgen, 2002). The increased energy expenditure associated with postprandial metabolism of protein and amino acids could contribute to protein-induced satiety, and the differential effects of protein source on satiety.

1.2 Breakfast protein and satiety

A high protein diet can be an effective method of weight management, but the timing of protein intake appears to make a critical difference in overall energy consumption, and many people are not eating their protein at the right time of day to have the largest satiety effect. Protein intake throughout the day is largely skewed towards the later meals of the day, with breakfast being the meal in which the lowest amount of protein was consumed; ~15-16% protein at breakfast compared with 31% at lunch and 40-42% at dinner (Rains, Maki, Fulgoni, & Auestad, 2013). However, this eating pattern may need reconsideration. Higher protein breakfasts have been associated with lower energy intake later in the day (Rains et al., 2013), as well as increased satiety (H. J. Leidy, Bossingham, Mattes, & Campbell, 2009). Because protein

at breakfast appears to have a stronger influence on satiety during later parts of the day, it is of interest to investigate the protein component of breakfast.

Egg white protein, an animal based protein source, is one of the highest quality proteins and considered to be easily digested earning it a full PDCAAS of 100. Being such an ideal protein source and a popular one consumed at breakfast time, I am interested in studying its effects on satiety when consumed at the first meal of the day. Unlike milk protein, there has been fewer studies looking at the digestive properties of egg white protein. Egg white protein has been demonstrated to be highly digestible, having a true digestibility of 98% in both human and rats (Bodwell, Satterlee, & Hackler, 1980). Egg white protein produced lower levels of total amino acids and protein nitrogen to urea conversion (an indirect measure of digestibility) when compared to the same amount of protein provided from cottage cheese, suggesting that egg white protein is a poorer protein choice compared to the casein and whey found in cottage cheese (Gannon, Nuttall, Lane, & Burmeister, 1992; Nuttall & Gannon, 1990). The digestive mechanism of egg white protein used in the study could have been compromised by the microwave cooking process which would alter the protein structure (Mine, Noutomi, & Haga, 1990). While egg white protein is considered to be a high quality protein source, it is uncertain how it compares to other protein sources in its satiety effects.

Similar to eggs, bread is a major component of breakfast for many individuals. Wheat gluten protein comes from the endosperms of grains used to produce bread and creates the elastic property of bread dough allowing it to rise during baking (Shewry, Halford, Belton, & Tatham, 2002). Wheat gluten protein, and other plant-based protein sources (i.e. rice, legumes), tend to contain less methionine and lysine compared to animal protein sources like egg white protein. Wheat gluten protein also contains considerably less essential amino acids than egg white protein

(Watson, 1996) and has a PDCAAS of 42 (G. Schaafsma, 2000). The satiety effect of wheat gluten protein compared to egg white protein has not been well characterized and has only been compared in two prior studies (Bayham et al., 2014; Lang et al., 1998). Lang and colleagues compared these two protein source with casein, gelatin, soy and pea protein, and found no difference in subsequent food intake between any of the protein sources (Lang et al., 1998). It may be likely that the 8 hr inter-meal interval before the buffet dinner provided was likely too long and any satiety effect may likely have returned to baseline by that time. Another study compared an "egg breakfast" to a "cereal breakfast" found the egg breakfast to increase fullness to a greater extent than the cereal breakfast (Bayham et al., 2014). In this study, preload meals had mixed protein sources which included milk and soy protein, as well as varied in fiber content. Better controlled studies are required to understand how egg white and wheat gluten proteins compare in satiety.

It was previously observed that rats given a 35% egg white protein meal at the first meal of the day were more satiated than when provided a 35% wheat gluten protein meal (Du, Markus, Fecych, Rhodes, & Lee Beverly, 2017). Additionally, rats given the 35% wheat gluten protein meal appeared less satiated than when given a meal containing 20% protein. These two protein sources are made up of different amino acid compositions with egg white protein being made up of more essential amino acids. This dissertation will investigate whether the satiety enhancing effects of egg white protein over wheat gluten protein is associated with different levels of circulating amino acids, insulin signaling, acute changes in whole animal metabolism and extended impact on whole animal metabolism and body weight. The central hypothesis of my dissertation is that egg white protein produces greater satiety than wheat gluten protein by

increasing circulating amino acids and satiety hormones, while also increasing energy expenditure.

1.3 Table

Table 1.1 Literature review of the effects of protein source on satiety. Ø, no difference; --, not assessed

Source	Subjects	Proteins tested	Protein amount	Energy	Meal type	Postprandial period	Outcome: Subsequent energy intake	Outcome: Perceived sensation (fullness, satiety, hunger)	Outcome: Hormonal	Outcome: Glucose, insulin, amino acids	Outcome: Metabolism
Semon et al. 1987 (USA)	30 rats	casein, lactalbumin, egg white, soy	40% protein	--	not specified (powdered?)	Food intake monitored at 15, 30, 90, 180 min	lactalbumin = casein > soy > egg	--	--	--	--
Uhe, Collier, and O'dea 1992 (Australia)	6 lean M	beef, chicken, fish	50 g	--	solid	15 min intervals for 3 hrs following preload	--	Satiety: Fish > beef = chicken	Insulin: Ø	Trp:AA Fish > beef = chicken; glucose Ø	--
Turnball, Walton, Leeds 1993 (UK)	13 lean F	Mycoprotein, chicken	44 g	570 kcal	solid	VAS at 1 hr intervals for 3 hrs. dietary recall for remainder of day and next day	chicken > mycoprotein	desire to eat: chicken > mycoprotein; hunger: Ø; fullness: Ø	--	--	--
Boirie et al. 1997 (France)	16 lean (gender?)	casein, whey	30g	--	beverage	Blood at 20 min interval for 7 hrs	--	--	--	Leu, total AA: Whey > casein (from 20-100 min), Casein > whey (from 300-420 min)	--
Lang et al. 1998 (France)	12 lean M	egg albumin, casein, gelatin, soy, pea, wheat gluten	22% of energy as protein	5.2 MJ	Mixed	Buffet dinner 8 hrs after preload lunch. VAS periodically for 12 and blood periodically for 8 hours.	Ø	Ø	Insulin, glucagon: Ø	Glucose: Ø	--

Table 1.1, continued

Source	Subjects	Proteins tested	Protein amount	Energy	Meal type	Postprandial period	Outcome: Subsequent energy intake	Outcome: Perceived sensation (fullness, satiety, hunger)	Outcome: Hormonal	Outcome: Glucose, insulin, amino acids	Outcome: Metabolism
Lang et al. 1999 (France)	9 lean M	Casein, gelatin, soy	23% (of which about 65% was experiment-tally manipulated)	3.6 or 1.8 MJ	Mixed	Buffet dinner 8 hrs after preload lunch. VAS periodically for 12 and blood periodically for 8 hours.	Ø	Ø	insulin, glucagon: Ø	Glucose: Ø	--
Mikkelsen, Toubro, Astrup 2000 (Denmark)	12 overweight and obese M	soy, pork	28%	430 kcal	solid	24 hr stay in respiratory chamber	Ø	--	--	--	24 h EE: pork > soy
Bensaid et al. 2002 (France)	rats	wheat gluten, total milk	50%	3 g	powdered	continuous food intake measurements	Ø	--	--	--	--
Hall et al. 2003 (UK)	16 lean healthy M/F	Casein, whey	48 g	1674-1695 kJ	beverage	20 min intervals for 3 hrs following preload. Buffet meal 90 mins following preload	Casein > whey	Desire to eat: casein > whey. Hunger and fullness: Ø	GIP, GLP-1, CCK: whey > casein	Val, Ile, Leu, Thr, BCAA: Whey > casein. Glucose, insulin: Ø	--
Anderson et al. 2004 (Canada)	Exp 1= 13 M; Exp 2 = 22 M	Exp 1: whey, soy, egg albumen; Exp 2: Whey, egg albumen	Exp2 = 50 g		beverage (400 mL)	Exp 1: one hour ad libitum pizza 1-2 hrs later	egg albumen=soy >whey	--	--	--	--
Bowen et al. 2006 (Australia)	72 lean and overweight M	Whey, soy, wheat gluten	50 g	1.1 MJ	beverage (450 mL)	Blood and VAS at 15 min intervals for 3 hrs following preload. Buffet lunch 3 hr following preload.	Ø	Ø	Ghrelin, CCK, GLP-1: Ø	glucose, insulin: Ø	--

Table 1.1, continued

Source	Subjects	Proteins tested	Protein amount	Energy	Meal type	Postprandial period	Outcome: Subsequent energy intake	Outcome: Perceived sensation (fullness, satiety, hunger)	Outcome: Hormonal	Outcome: Glucose, insulin, amino acids	Outcome: Metabolism
Bowen et al. 2006 (Australia)	19 overweight M	Casein, whey	55 g	1069-1090 kJ (2.7 kJ/g)	beverage	Blood and VAS at 15 min intervals for 3 hrs following preload. Buffet lunch 3 hr following preload.	Ø	Ø	CCK, ghrelin: Ø	glucose, insulin, AA: Ø	--
Pichon et al. 2008 (France)	rats	whey, whey w/ B-lactoglobulin, milk	55%		moistened powdered diet	ad libitum	Milk > whey = Blac	--	leptin, insulin: milk=whey > B-lac	glucose: Ø	--
Diepvens, Haberer, Westerterp-Plantenga 2008 (Netherlands)	39 lean M/F	Whey, pea protein hydrolysate, milk (80:20 casein:whey)	15 g (25%)	1024 kJ	beverage	Blood at 30 min interval for 2 hrs. VAS at 30 min intervals for 4 hrs. Buffet lunch 3 hrs following preload	Ø	Hunger: milk > pea (90 min), whey > pea (240 min); Satiety: pea > milk (180 min)	CCK: milk > whey > pea; GLP-1: milk > whey=pea; PYY:Ø; Ghrelin:Ø	insulin Ø; glucose Ø	--
Veldhorst et al. 2009 (Netherlands)	25 lean M/F	casein, soy, whey	10 & 25%	4 kJ/g (20% of daily energy require ments, 2.52 MJ)	custard	VAS and AA for 4 hrs. Glucose and hormones for 3 hrs. Buffet lunch 3 hrs following preload.	Ø	at 10% PRO, hunger: casein = soy > whey; at 25% PRO, Ø	at 10% PRO, ghrelin: soy > casein; at 25% PRO, GLP-1: Whey > casein	at 10% PRO, Leu, Lys, Trp, Ile, Thr: whey > casein = soy, insulin: casein > soy; at 25% PRO, Insulin: whey > casein = soy, glucose: soy > casein	--
Alfenas et al. 2010 (Brasil)	26 lean M/F	casein, soy, whey	0.5 g/kg bodyweight	265 kcal	milk shake w/ crackers or cookies or cake	VAS at 60 min interval for 2 hours	whey > casein	Ø	--	--	DIT on day 7: soy > whey; RQ: soy > whey

Table 1.1, continued

Source	Subjects	Proteins tested	Protein amount	Energy	Meal type	Postprandial period	Outcome: Subsequent energy intake	Outcome: Perceived sensation (fullness, satiety, hunger)	Outcome: Hormonal	Outcome: Glucose, insulin, amino acids	Outcome: Metabolism
Pal and Ellis 2010 (Australia)	22 lean M	whey, tuna, turkey, egg albumin	50 g (71%)	1200 kJ	beverage (600 mL)	blood at 30 min interval for 4 hrs. buffet lunch 4 hrs following preload	egg = turkey > tuna > whey	hunger: tuna = turkey = egg > whey; fullness: whey = tuna > egg = turkey	--	glucose: Egg > turkey > whey; insulin: whey > egg = turkey = tuna	--
Abou-Samra R et al. 2011 (Switzerland)	32 lean M	pea, casein, whey, egg albumin	20 g	80 kcals	beverage (250 mL)	30 minutes prior to ad lib meal. VAS at 10 minute intervals for one hour	Ø	combined satiety score: casein = pea > egg = whey	--	glucose: Whey < pea=casein=egg	--
Acheson et al. 2011 (Switzerland)	23 lean adult M/F	whey, casein soy	50%	459 kcal (energy density 1 kcal/g)	beverage	Continuously collect appetite rating, blood samples, indirect calorimetry for 330 mins following test meal	--	Satiety casein = soy > whey; Hunger whey > casein=soy	--	insulin Ø; glucose Ø	Energy expenditure (5.5 hrs), whey>casein =soy; Thermic effect whey>casein =soy
Munsters, Geraedts and Saris 2013 (Netherlands)	10 lean M	dairy (70% dairy, 30% vegetable) vs vegetable (gluten, nut, soy) protein	15%	Inter-vention through out day	Solid	Blood collected 30, 60, 120, 240 following each of 3 meals. Glucose and indirect calorimetry measured continuously for 36 hrs. VAS 60 mins following meal.	--	Ø	GLP1: Ø	Glucose: Dairy > vegetable (at some sampling points) ; Insulin: Ø	Sleeping metabolic rate: Vegetable > dairy; DIT: Ø; TEE: Ø

Table 1.1, continued

Source	Subjects	Proteins tested	Protein amount	Energy	Meal type	Postprandial period	Outcome: Subsequent energy intake	Outcome: Perceived sensation (fullness, satiety, hunger)	Outcome: Hormonal	Outcome: Glucose, insulin, amino acids	Outcome: Metabolism
Bendtsen et al. 2014 (Denmark)	24 overweight/ obese M/F	casein (intact and hydrolyzed) and whey (intact)	30 g (26%)	20% of daily energy require ments	beverage	Blood collected at 15, 30, 60, 90, 120, 180, 240 min. Buffet lunch 3 hrs following preload	Ø	Ø	GLP-1: Ø	Insulin: Ø	24 h EE: Ø; postprandial EE: Ø; lipid oxidation: intact whey > hydrolyzed casein
Bayham et al. 2014 (USA)	20 overweight/ obese M/F	Egg vs. cereal breakfast (both mixed protein)	19.80%	400 kcal (1.37 kcal/g)	solid	180 mins prior to ad lib meal. Blood: 30 , 60, 120, 180 min following preload	Ø	Fullness egg BF > cereal BF	PYY: egg > cereal; ghrelin: cereal > egg	Glucose, Insulin, Leu: Ø	--
Douglas, Lasley, Leidy 2015 (USA)	21 lean M/F	beef, soy	24 g (34 g overall)	400 kcal	solid	VAS and blood collected at 30 min intervals for 7 hrs. Buffet meal when requested by subjects	Ø	Ø	Ø	--	--
Crowder, Neumann, Baum 2016 (USA)	20 lean and overweight F	animal (egg white, turkey, dairy), plant (soy)	26-27 g	375 kcal	solid	VAS and blood glucose at 15 min intervals for 2 hours. 24 hr food recall	Ø	Ø	--	glucose: plant > animal	--
Kristensen et al. 2016 (Denmark)	43 lean M	Vegetable (beans, peas) vs. animal (veal, pork)	19%	3.5 MJ	solid	VAS at 30 min intervals for 3 hrs. Buffet meal 3 hrs after preload	meat > legume	Satiety: legume > meat; hunger: meat > legume	--	--	--

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Chapter 2: Effect of protein quantity and source on postprandial plasma amino acid levels

2.1 Introduction

A potential explanation for the observed difference in satiety following ingestion of a meal containing either egg white or wheat gluten protein is differences in blood levels of amino acids that each protein source induces. Protein meals increase plasma amino acid levels and the detection of these amino acids by the body contributes to the perception of satiety (Boirie et al., 1997; Hall, Millward, Long, & Morgan, 2003; M. A. Veldhorst et al., 2009b; M. A. B. Veldhorst, A. G. Nieuwenhuizen, A. Hochstenbach-Waelen, K. R. Westerterp, et al., 2009). According to the aminostatic hypothesis, when plasma levels of amino acids reach beyond the amount required for protein synthesis, excess amino acids serve as a satiety signal in the brain to decrease food intake (Mellinkoff, Frankland, Boyle, & Greipel, 1956). Consistent with the aminostatic hypothesis, higher protein meals which increase plasma amino acids to a greater extent than lower protein meals induce greater satiety all else being equal (M. A. Veldhorst et al., 2009b; M. A. B. Veldhorst, A. G. Nieuwenhuizen, A. Hochstenbach-Waelen, K. R. Westerterp, et al., 2009). Additionally, different sources of proteins have also been demonstrated to have unique postprandial plasma amino acid profiles (Boirie et al., 1997; Hall et al., 2003; Uhe, Collier, & O'Dea, 1992; M. A. B. Veldhorst, A. G. Nieuwenhuizen, A. Hochstenbach-Waelen, A. J. A. H. van Vught, et al., 2009). The uniqueness of food matrixes of different protein sources contributes to rates of digestion and accordingly affects amino acid absorption. This has been shown with the ingestion of equal amounts of casein and whey proteins. A single meal containing whey protein caused a quick, steep increase in plasma amino acid levels that was rapidly decreased to baseline (Boirie et al., 1997). On the other hand, plasma amino acid levels following casein protein ingestion increased slowly and remained at an increased plateau for

over five hours following ingestion. These plasma amino acid results correspond to observations of increased satiety soon after ingestion of whey protein (Bendtsen, Lorenzen, Bendsen, Rasmussen, & Astrup, 2013). Hence, even at isocaloric levels, different protein sources can affect satiety by causing distinctive patterns in absorption of amino acids.

Concentration of plasma amino acids from dietary protein also depends on various complex factors involved in splanchnic tissue absorption and oxidation. Amino acids are absorbed from the lumen of the intestine through transporters located on the apical and basolateral membranes of the intestinal epithelial cells that are specific to properties of each amino acid. Absorption rate of specific amino acids through distinct amino acid transport systems depends on the gradient of amino acid present in the lumen (Bröer, 2008). In addition to variable rates of intestinal absorption, plasma amino acid concentration will also depend on uptake for catabolism by the liver and other tissues (Rerat, Simoes-Nunes, Mendy, Vaissade, & Vaugelade, 1992). Enterocytes heavily rely on amino acids as a primary source of fuel, with >90% of glutamine, glutamate and aspartate from dietary protein catabolized by first-pass metabolism from the small intestine (Reeds, Burrin, Stoll, & van Goudoever, 2000; Wu, 1998). Of the remaining amino acids, intestinal degradation accounts for 30-50% reduction in availability to other tissues (Wu, 1998, 2009). Liver metabolism of amino acids further modifies the pool of free amino acid in circulation with the exception of branch chain amino acids (BCAA), leucine, isoleucine, and valine, which do not get metabolized by the liver (Wu, 2009). Rate of absorption and first-pass metabolism play extensive roles in modulating availability of amino acids from dietary proteins.

Different protein sources vary in their amino acid composition which will then effect intestinal absorption and appearance in plasma. As an animal source of protein, the amino acid

composition of egg white protein varies substantially from wheat gluten protein, a plant based protein source. Wheat gluten protein is considered "prolamins" for its characteristically high levels of glutamine and proline (Bos et al., 2005). The increased levels of these two amino acids come at the expense of indispensable amino acids, including valine, isoleucine, leucine, lysine, aspartate, alanine, and methionine (Watson, 1996). Several amino acids, including leucine, tryptophan, lysine, and arginine have been suggested to have direct satiety signaling functions (Daniela Cota et al., 2006; Fernstrom, 1985; Jordi et al., 2013; Layman & Baum, 2004; Nieuwenhuizen et al., 2009; M. A. Veldhorst et al., 2009a; Zhang et al., 2007). Leucine is suggested to signal satiety by activation of mammalian target of rapamycin (Daniela Cota et al., 2006; Woods, Seeley, & Cota, 2008). Oral infusions of arginine, lysine, and glutamate has been demonstrated to decrease subsequent food intake (Jordi et al., 2013). Further investigations are necessary to understand the satiety signaling role of individual amino acids.

It is important to understand how postprandial plasma amino acid profiles differ following consumption of various protein source because of the implications for protein-induced satiety. Postprandial changes in amino acid levels that correspond with enhanced satiety can provide indications of amino acids critical to the satiety signaling mechanism. Concentrations of plasma amino acids following ingestion of meals in which the protein source came solely from egg white and wheat gluten protein have yet to be reported. The goal of this chapter was to quantify postprandial changes in plasma concentration of amino acids in response to wheat gluten versus egg white meals to determine the extent to which the patterns were correlated with the previously established satiety responses. It is hypothesized that egg white protein would yield higher levels of amino acids in general because of differential absorption, and that specific amino

acids including leucine, lysine, and arginine, suggested from the literature to induce satiety, would be increased relative to wheat gluten.

2.2 Methods

Animals. Male Sprague-Dawley rats (approximately 200 g; Charles River Laboratories) were singly housed in plexiglass cages (30 x 30 x 38 cm). The floors of the cages were fitted with steel wired bottoms resting approximately one inch above the cage floor in order to measure diet spillage. Animals were kept in a temperature-controlled room (26 ± 2 ˚C) and given free access to water. Upon arrival to our research facility, animals were trained to a reversed 12 h light:dark cycle (lights off at 800) and to a meal-feeding schedule. Acclimation to the diets and feeding schedule was designed to be a gradual process over 10 days. The process started with pelleted rodent chow (Harlan Teklabs, Madison, WI) being replaced with ground chow, food provided *ad libitum* for 10 hours of the dark cycle and progressed into feeding test diets at restricted meal times. Food was removed one hour prior to the onset of the light cycle for an overnight fast. At one hour into the dark cycle (900 hr), animals were given 30 minutes to consume a meal, representing 20% average daily intake by weight (average of food intake on previous 3 days). Ground chow was available ad libitum between 1330 and 1900 hr. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois and are in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council).

Diet preparation. Diets were prepared to be comparable to AIN-93G (Reeves, 1997). Separate mineral mixes were used to account for micronutrient differences in the egg white powder and wheat gluten powder. All diet components were purchased from Dyets, Inc. (Bethlehem, PA), with the exception of egg white powder and maltodextrin which were purchased from Harlan

(Indianapolis, IN). The two higher protein diets were approximately 45:35:20 (carbohydrate:protein:fat, as a % of calories) with protein provided by either egg white protein or wheat gluten protein. The two normal protein diets were approximately 60:20:20 (carbohydrate:protein:fat, as a % of calories) with protein provided by either egg white protein or wheat gluten protein.

Surgical procedure. Rats were acclimated for a one-week period prior to surgical procedure. Rats were anesthetized with isoflurane. Once anesthetized, the top of the head and the neck is shaved and the skin prepared with Povidone-Iodine 10%. A small (<3 cm) vertical incision is made in the neck to access a segment of the right jugular vein. A sterile Silastic catheter (0.025 in. ID x0.047 in. OD) filled with heparinized saline is inserted into the vein until reaching the right atrium, it is pulled back from the atrium for placement in the sinus of the superior vena cava. The catheter is secured to the vein with suture, tunneled under the skin and externalized through an incision made on the top of the head. The incision on the neck is closed with sterile wound staples. Patency of the catheter was maintained by filling with a 90% glycerol solution (v/v) containing 500 U heparin/ml and capped with a sealed piece of Tygon tubing. Rats are then placed into a stereotaxic instrument (ASI Instruments, Warren, MI) and end of the catheter fixed in position with dental acrylic cement and anchored to the skull with four stainless steel screws. Following surgery, rats were monitored until they were fully recovered from the anesthesia and postsurgical analgesia provided by carprofen (5 mg/kg body weight s.c.) on the day of surgery and two days afterwards. Animals were evaluated daily for at least 7 days post-surgery.

Sample collection. Rats were given 5-7 days of ad libitum food access after surgery in order to regain lost weight and recover from the operation. Catheter patency was maintained by flushing catheters with heparinized 0.9% saline solution and filling them with 90% (v/v) glycerol solution

containing 500 U/mL heparin. The flushing protocol was performed daily for 3 days following surgery and every other day afterwards. Only animals with body weights greater than on the day of surgery were used for sample collection. Once fully recovered, the feeding regimen resumed and the blood sampling protocol was initiated. On the day of sample, a 20 inch Tygon tube is connected to the externalized portion of the catheter at least one hour prior to sample collection. The other end of the tube is weighted on the outside of the cage so that collections can be made from unrestrained animals. A total of 6 samples were taken every 30 minutes, with the first sample taken 15 minutes before the test diet was given and the final sample taken 120 minutes (2 hours) after the test diet was given. Approximately 250 microliters of whole blood were collected at each time point and placed into heparinized microtubes. Each sample was briefly centrifuged for 10-15 seconds at 6000 rpm and 100 microliters of plasma were harvested. Blood volume was maintained by resuspending harvested red blood cells in a volume of 0.9% sterile saline solution equal to the amount of plasma removed at each time point. The resuspended red blood cells were readministered to each animal through the jugular vein following collection of the next sample. Plasma samples were kept on wet ice through sample collection and kept at -80 degree Celsius until amino acid analysis.

HPLC amino acid analysis. Amino acids were analyzed by HPLC on a Dionex WPS-3000 system (Thermo Scientific). Supernatant from plasma mixed with 3.5% perchloric acid and centrifuged for 10 minutes at 8000 rpm was injected onto a 250 x 4.0 mm C18 (5 µm) column after derivatization in an o-phthaldialdehyde solution. Amino acids were separated using a binary gradient of 10 mM Na2HPO4, 10 mM Na2B4O7·10H20 (pH 7.8) and acetonitrile/methanol/water (45/45/10 v/v/v) and quantified by flourometric detection (excitation:

337 nm, emission: 442 nm). Data were collected and analyzed using Chromeleon Data System software (Thermo Fischer).

Statistical Analysis. Data were analyzed using SAS v9.4 (SAS Institute Inc., Cary, NC). Concentrations of amino acids were analyzed by repeated measures analysis with time point (6 levels) as the within-subject factor and Treatment (20EW, 20WG, 35EW, 35WG) as the between-subject factor. When treatment effects were detected without an effect of interaction of treatment and time point, data were collapsed across time-points and these values were analyzed by 2-way ANOVA with concentration (20% or 35% protein), type (egg white or wheat gluten), and their interaction as factors. The sum of these ten measured amino acids were also analyzed by repeated measures two-way ANOVA with time point as the within subjects factor, and protein type as the between-subjects factor. Tukey's least square difference (t-tests, with mean square error from ANOVA model) were used to evaluate pair-wise differences between means. Values were presented as means ± SEM. An alpha level of $P<0.05$ was considered statistically significant.

2.3 Results

A total of ten amino acids were measured, seven of which are considered indispensable amino acids (lysine, methionine, leucine, isoleucine, valine, phenylalanine, tryptophan) and three non-essential amino acids (cysteine, alanine, and tyrosine) (Figure 2.1 and 2.2). Significant effect of time indicated that most of the measured amino acids increased from baseline collapsed across treatment groups (all p-values <0.05), with the exception of methionine and tryptophan. Analysis of the sum of these ten measured amino acids revealed significant differences between treatment groups ($F_{3,12}=11.53$, $P<0.001$), but no significant interaction between treatment group and time point ($F_{15,54}=1.39$, $P=0.1841$) (Figure 2.1a). Posthoc analysis revealed increased total amino

acids following 35EW compared to 35WG (P=0.029), and 20EW compared to 20WG (P=0.004). A two-way ANOVA of time point by protein type indicated a significant main effect of time ($F_{5.39}$=5.86, P<0.001), protein type ($F_{1,5}$=28.05, P=0.003), and interaction of time with protein type ($F_{5,24}$=3.10, P=0.027) collapsed across protein concentrations. Amino acids peaked immediately after the wheat gluten meal at time point 0 min, whereas amino acids peaked at timepoint 30 min after the egg white meal. Posthoc comparison indicated overall amino acids following wheat gluten meals differed from baseline at 0 min (P=0.002), and no other differences from baseline were detected. Overall amino acids following egg white meals differed from baseline at 30 min, 60 min, 90 min, and 120 min (all p-values <0.01). Two-way ANOVA collapsed across time points showed a main effect of protein type ($F_{1,12}$=15.19, P=0.002) indicating 37% increased overall amino acids in egg white treatments compared to wheat gluten treatments. Concentration of protein was not significant ($F_{1,12}$=1.24, P=0.287).

Measurements of individual amino acids were also summed into branched chain amino acids (BCAAs) (Figure 2.1b). There was a treatment effect detected in measurements of BCAAs ($F_{3,12}$=17.23, P<0.001), as well as an interaction between treatment group and timepoint ($F_{15,54}$=2.07, P<0.026). Concentrations of BCAAs following 20EW (P=0.016), 35WG (P>0.001), and 35EW (P>0.001) were greater than following 20WG ingestion. The interaction effect is a result of BCAAs in the 20WG remaining close to baseline levels for the two hours during which blood samples were collected compared to the other treatment groups which increased from baseline.

Significant differences between treatment groups were apparent in concentrations of lysine ($F_{3,12}$=31.44, P<0.001), tyrosine ($F_{3,12}$=11.78, P<0.001), isoleucine ($F_{3,12}$=14.77, P<0.001), valine ($F_{3,12}$=27.00, P<0.001), phenylalanine ($F_{3,12}$=8.04, P<0.005), leucine ($F_{3,12}$=14.22,

P<0.001), and tryptophan ($F_{3,12}$=17.56, P<0.001). No significant differences between diet treatments were detected in postprandial concentrations of alanine and cysteine. Egg white protein caused a greater postprandial lysine, valine, tryptophan, and isoleucine concentrations compared to wheat gluten protein at both 20% and 35% protein. Posthoc analysis revealed that 35EW caused greater lysine (P<0.001), valine (P=0.004), and tryptophan (P=0.015) compared to 35WG. At the lower concentration of dietary protein, 20EW caused increased levels of lysine (P<0.001), valine (P=0.001), and tryptophan (P=0.005), as well as isoleucine (P=0.047), when compared to 20WG. The two higher protein meals, 35EW and 35WG, showed increased postprandial concentrations of several amino acids compared to 20WG. This was indicated by posthoc analysis of treatments collapsed across time showing lower concentration following 20WG when compared to 35EW and 35WG for tyrosine (P=0.001 and P=0.002), isoleucine (P<0.001 and P<0.001), valine (P<0.001 and P=0.001), phenylalanine (P=0.007 and P=0.004), leucine (P=0.006 and P<0.001), and tryptophan (P=0.001 and P=0.002).

Interactions between treatment group and time point were detected for lysine ($F_{15,54}$=2.88, P=0.002), isoleucine ($F_{15,53}$=1.89, P=0.045), and valine ($F_{15,54}$=3.05, P=0.001). In the case of valine and isoleucine, the interaction effect is a result of valine and isoleucine in the 20WG group remaining close to baseline levels for the two hours during which blood samples were collected compared to the other treatment groups which increased from baseline (Figure 2.2a,e). Posthoc comparisons indicated isoleucine and valine following 20WG did not differ from baseline relative at any time points, whereas levels did differ from baseline following 20EW, 35WG, and 35EW (all p-values <0.01). The interaction effect in lysine is a result of decreasing lysine following wheat gluten treatments and increasing lysine following egg white treatments when compared to baseline values (Figure 2.1f). This is indicated by two-way ANOVA of time

point by protein type indicating a significant main effect of time ($F_{5.39}$=4.86, P=0.002), protein type ($F_{1,5}$=83.04, P<0.001), and interaction of time with protein type ($F_{5,24}$=6.21, P<0.001) collapsed across protein concentrations. Posthoc comparisons indicated lysine following egg white increased from baseline at time point 30 and 60 min (both p-values <0.01), whereas levels decreased from baseline following wheat gluten at 60, 90, and 120 min (all p-values <0.05).

Two-way ANOVA collapsed across time points showed a main effect of protein concentration for tyrosine ($F_{1,12}$=7.05, P=0.021), phenylalanine ($F_{1,12}$=5.95, P=0.031), and tryptophan ($F_{1,12}$=7.46, P=0.018) (Figure 2.1e, 2.2c, 2.2d). This indicates 27% increase in tyrosine, 19% increase in phenylalanine, and 21% increase in tryptophan in the 35% protein group compared to the 20% protein group. Type of protein was also significant for tryptophan ($F_{1,12}$=9.41, P=0.010), indicating 24% increase in tryptophan in egg white treatments compared to wheat gluten treatments. Type of protein was not significant for tyrosine ($F_{1,12}$=0.74, P=0.406) and phenylalanine ($F_{1,12}$=0.54, P=0.475).

2.4 Discussion

This study demonstrates that rats fed meals containing isoenergetic amounts of protein coming from different sources of protein have unique postprandial plasma profiles of amino acids. The main findings from this study were that egg white protein meals resulted in greater levels of overall plasma amino acids compared with wheat gluten meals. Additionally, analysis of postprandial plasma for individual amino acids revealed greater increases in lysine, isoleucine, valine, and tryptophan following ingestion of egg white protein compared with wheat gluten protein at both the 35% and 20% protein level. In a prior study, in which a control diet was made available 15 minutes following ingestion of the test meal, subsequent food intake data showed that rats fed 35EW consumed a smaller subsequent meal, an indication of satiety, compared with

rats fed 35WG. In that study, rats ingested the subsequent meals within the hour after the time allotted for consumption of the test meal. The increased postprandial plasma amino acids found in the present study occurred during the 2 hours following ingestion of the different protein meals, coinciding with the duration of the subsequent meal in the prior study. Overall, these results support the involvement of the increased circulation of amino acids in protein-induced satiety.

This study adds further evidence to the findings from other studies demonstrating that ingestion of isoenergetic amounts of protein from different protein sources can cause unique patterns of plasma amino acid appearances (Boirie et al., 1997; Hall et al., 2003; Uhe et al., 1992; M. A. B. Veldhorst, A. G. Nieuwenhuizen, A. Hochstenbach-Waelen, A. J. A. H. van Vught, et al., 2009). Furthermore, these data support the idea that differences in protein-induced satiety between protein sources may be dependent on the capacity of protein sources to release amino acids into circulation. We previously found egg white protein meals to cause decreased subsequent food intake, and the results in this study showed egg white protein meals to increase overall amino acids to a greater level than wheat gluten meals. Other studies have also reported findings that support increased amino acids in circulation to correspond with decreased food intake and increased satiety (Hall et al., 2003; Mellinkoff et al., 1956; M. A. Veldhorst et al., 2009b; M. A. B. Veldhorst, A. G. Nieuwenhuizen, A. Hochstenbach-Waelen, K. R. Westerterp, et al., 2009). Wheat gluten protein appears to have poorer bioavailability compared to egg white protein, which is especially evident at the 20% protein level. While the other three treatment meals caused increased postprandial plasma amino acids, several of the measured amino acids stayed near baseline levels following 20WG ingestion. This suggests that increased amounts of wheat gluten protein is needed to cause greater changes in postprandial plasma amino acid

levels. The results of this study demonstrate that even though the meals are isoenergetic in protein content, protein source plays a large factor in altering blood amino acid levels. Increased amino acid availability of egg white protein is likely contributing to satiety following ingestion of meals containing egg white protein.

Not only does overall amino acid levels differ following ingestion of egg white and wheat gluten protein containing meals, but the kinetics of amino acid availability also varies between the two protein sources. Ingestion of meals containing egg white protein produces a slow increase in amino acids, which peaks at 30 and 60 minutes for 35EW and 20EW, respectively, and remains elevated from baseline concentration for the 2 hour blood sampling duration (Figure 2.1a). On the other hand, ingestion of wheat gluten meals caused an almost immediate peak in amino acid concentrations. This peak is followed by plasma amino acid quickly returning to baseline values within 30 minutes of meal ingestion. Several other studies done in humans have attributed the increased satiety following ingestion of whey protein meal in comparison with isoenergetic casein protein meal to be due to the quick and sharp increase in plasma amino acid concentrations following the meal containing whey protein meal (Boirie et al., 1997; Hall et al., 2003). However, the time course of the changes in plasma amino acid concentration in those studies differed from the post-meal period measured in the present study. These other studies measured plasma amino acids for 3 or 7 hours following meal ingestion and saw the greatest increases in plasma amino acids over the initial 60 minutes. In our study, overall amino acid levels peaked immediately following the meal in wheat gluten protein fed rats and by 30 minutes in egg white protein fed rats. Therefore, the pattern of plasma amino acid increase observed following egg white protein ingestion in our study bears a closer resemblance to that of whey protein ingestion in other studies. Egg white protein meals cause a steadier rise of plasma amino

acid that remains elevated, which is likely supporting subsequent satiety and decreased food intake.

Aside from overall plasma amino acid concentrations, these results also revealed differences in several specific amino acids. Both egg white protein treatment meals caused a greater in lysine, tryptophan, isoleucine, and valine than the wheat gluten meals. My prior findings demonstrated that egg white protein induces a greater satiety response. Therefore, taken together, results are consistent with another study demonstrating a gavage of lysine to be among one of the most potent amino acids to reduce food intake in rats (Jordi et al., 2013). Their study also found arginine and glutamate to decrease subsequent food intake, two amino acids that were not measured in our study. Contrary to my finding that satiety corresponds with increased isoleucine, valine, and tryptophan, they did not identify these amino acids to be anorexigenic amino acids. A separate study found that rats given *ad libitum* access to diets supplemented with 5% lysine and tryptophan had reduced food intake (Ayaso, Ghattas, Abiad, & Obeid, 2014). Their study did not have a valine or isoleucine treatment group. Other studies in humans have demonstrated the increased satiety from ingestion of a whey protein meal coincided with higher concentrations of lysine, tryptophan, isoleucine, and valine (Hall et al., 2003; M. A. B. Veldhorst, A. G. Nieuwenhuizen, A. Hochstenbach-Waelen, A. J. A. H. van Vught, et al., 2009). The increased concentration of lysine, tryptophan, isoleucine, and valine following ingestion of the more potent satiety-inducing egg white protein meals, suggests their potential involvement in inducing satiety. Future research is needed to explore these four amino acids further to establish whether they play a mechanistic role in satiety.

Postprandial changes in plasma leucine have been implicated in promoting satiety. However, in our study, postprandial plasma leucine levels did not reflect satiety responses from

prior studies. While we have previously observed differences in satiety between 35EW and 35WG, leucine levels did not differ between these two treatment groups. These results are in opposition of the leucine-satiety hypothesis. This is consistent with other reports which failed to demonstrate correlations between physiological levels of plasma leucine and food intake (D. Cota et al., 2006; Layman, 2003; Zhang et al., 2007). While the egg white protein contains slightly higher levels of leucine compared to wheat gluten protein, this did not translate into higher postprandial circulating levels of the amino acid. It is possible that greater concentrations of leucine than were used in our study are needed to produce the satiety enhancing effects of this amino acid. Although the role of leucine in inducing satiety should not be discounted, its role may be less important than is suggested based on findings from other studies.

Our plasma amino acid analysis protocol was limited to the detection of ten amino acids out of the twenty, leaving the possibility that there could be differences in other amino acids that were not analyzed in this study. However, if a satiety mechanism were dependent on a few select amino acids, it likely would depend on the sensing of essential amino acid, a majority of which are accounted for in this study. This is because essential amino acids are those amino acids that cannot be produced by the body, they must be obtained through dietary sources, and as such would better indicators of satiety. Of the essential amino acids measured, none were found to be greater following ingestion of wheat gluten meals in comparison to isoenergetic egg protein meals. Overall, these data are consistent with the idea that increased essential amino acids in circulation following egg white protein meals are likely involved in inducing satiety.

To conclude, the data collected from this study support the hypothesis that greater increases in overall amino acids following a meal of egg white protein are associated with satiety signaling. Furthermore, greater increases in several amino acids, including lysine, isoleucine,

valine, and tryptophan following egg white meals, correspond to increased satiety following egg white meals. It would be worthwhile in future studies to explore the extent at which these amino acids may contribute to satiety signaling.

2.5 Figures

Figure 2.1

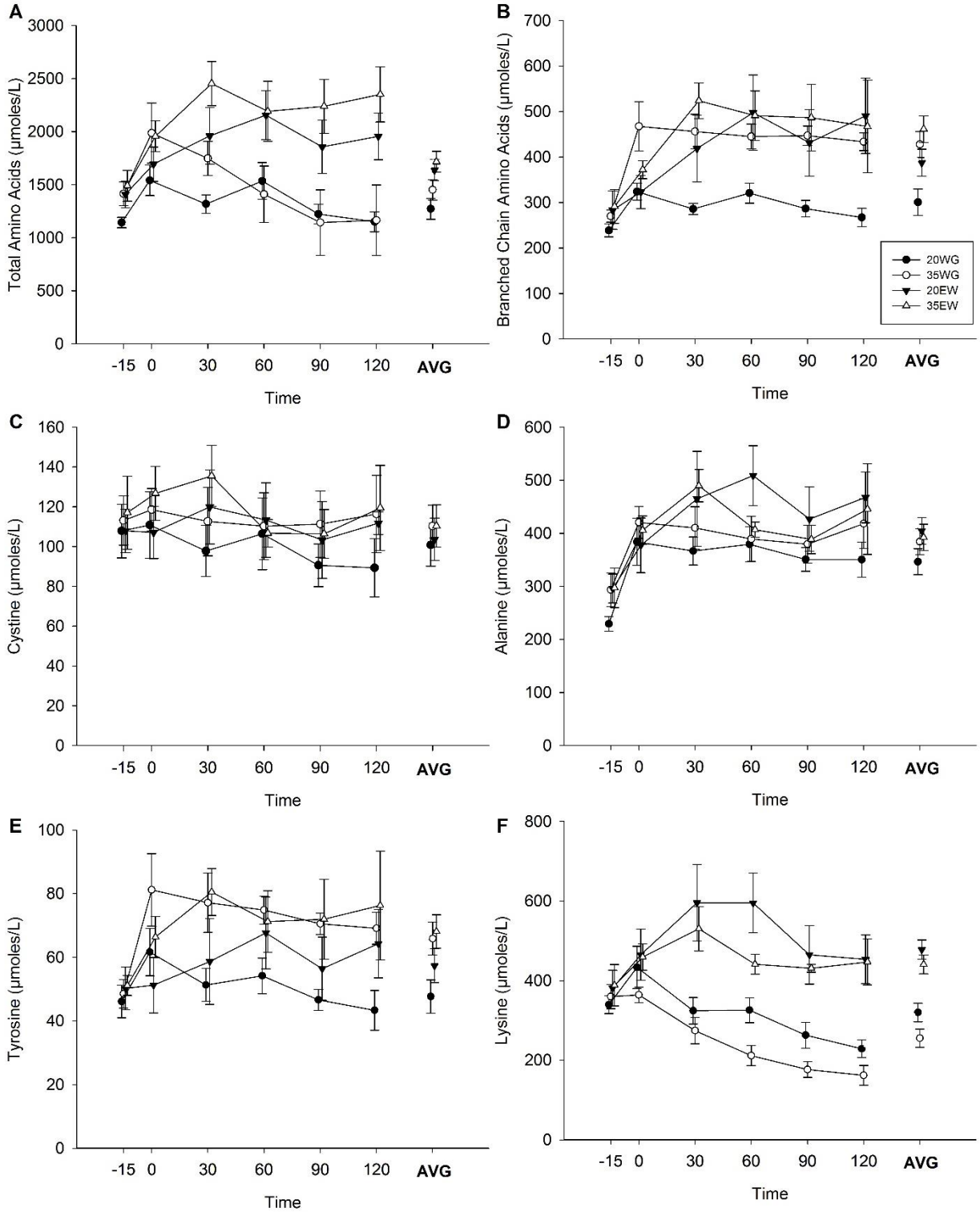

Figure 2.1 (continued). Effect of treatment meal on postprandial plasma amino acid concentrations. (A) Average concentration of total amino acids ± SEM at each time-point, 15 minutes before the test meal, at the end of the test meal (time-point 0), and 30 min increments thereafter. Following the 120 min time-point, the least square mean (AVG) collapsed across all time-points is shown with the standard error from the ANOVA model. (B) Same as A for branched-chain amino acids (leucine + lysine + valine). (C) Same as A for cystine. (D) Same as A for alanine. (E) Same as A for tyrosine. (F) Same as A for lysine. *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

Figure 2.2

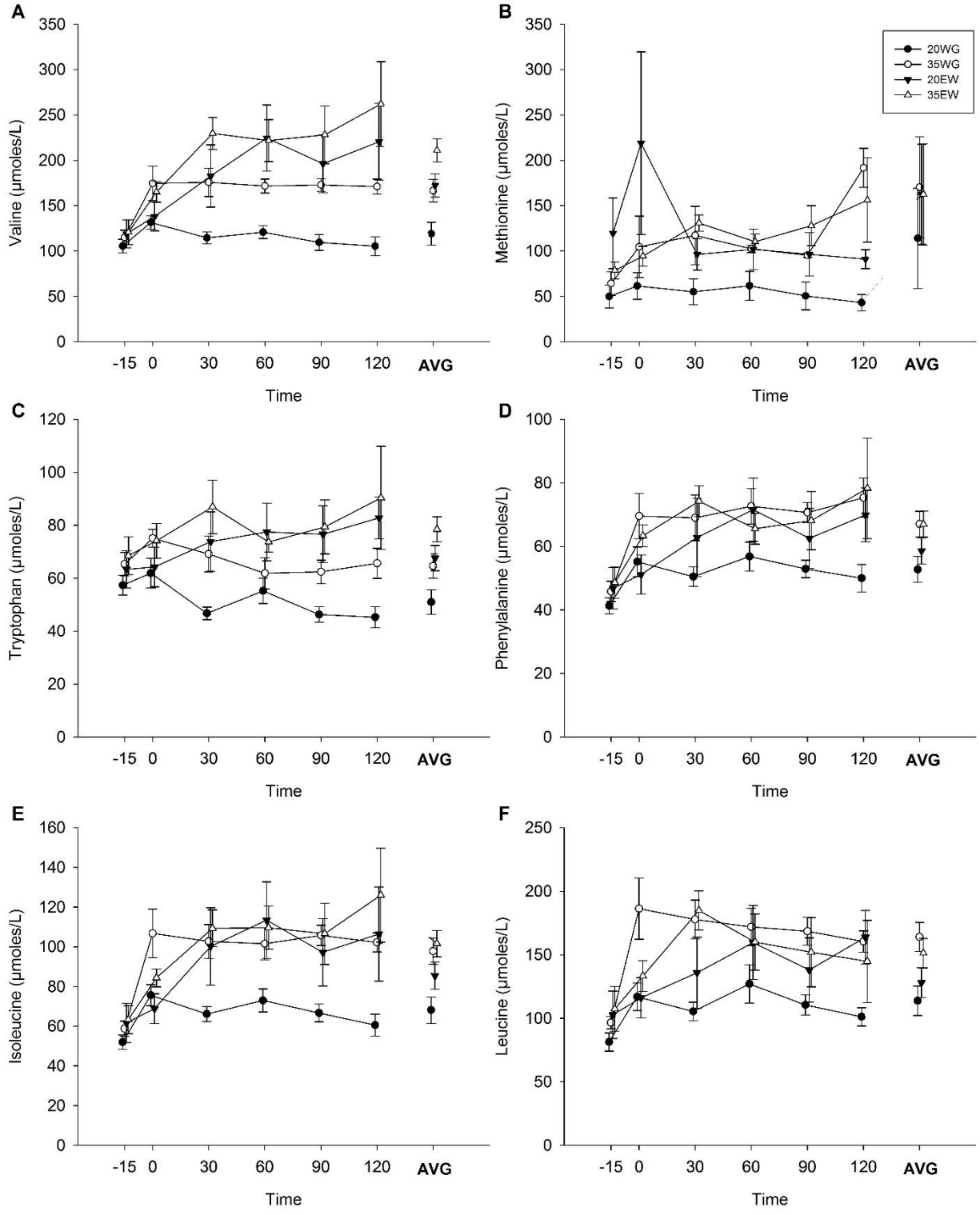
A
Valine (µmoles/L)
Time
B
Methionine (µmoles/L)
20WG
35WG
20EW
35EW
C
Tryptophan (µmoles/L)
D
Phenylalanine (µmoles/L)
E
Isoleucine (µmoles/L)
F
Leucine (µmoles/L)
-15 0 30 60 90 120 AVG

Figure 2.2. (continued). Effect of treatment meal on postprandial plasma amino acid concentrations. (A) Average concentration of total valine ± SEM at each time-point, 15 minutes before the test meal, at the end of the test meal (time-point 0), and 30 min increments thereafter. Following the 120 min time-point, the least square mean (AVG) collapsed across all time-points is shown with the standard error from the ANOVA model. (B) Same as A for methionine. (C) Same as A for tryptophan. (D) Same as A for phenylalanine. (E) Same as A for isoleucine. (F) Same as A for leucine. *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

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Chapter 3: Insulin response to meal ingestion of varying protein source and quality

3.1 Introduction

Another potential explanation for the differential satiety induced from egg white protein as compared to wheat gluten protein is the hormonal response and subsequent impact on food intake. The control of food intake depends on the integration of multiple systems for detecting energy availability. One of the crucial hormones involved in energy detection and processing is insulin. In many carbohydrate dominant foods, there is a strong correlation between postprandial glucose response (glycemic index, GI) and insulin secretion (S. Holt, Miller, & Petocz, 1997). However, protein ingestion weakens the relationship because protein foods cause greater insulin secretion than would be expected for the low glycemia that is produced (Liljeberg & Björck, 2001; Nilsson, Stenberg, Frid, Holst, & Björck, 2004; Spiller et al., 1987). Protein co-ingested with carbohydrate produces a synergistic effect on insulin secretion, producing a greater release of insulin than carbohydrate alone would (Calbet & MacLean, 2002; Nuttall, Gannon, Wald, & Ahmed, 1985; Nuttall, Mooradian, Gannon, Billington, & Krezowski, 1984; Pallotta & Kennedy, 1968; Rabinowitz, Merimee, Maffezzoli, & Burgess, 1966; van Loon, Saris, Verhagen, & Wagenmakers, 2000). Postprandial increases in insulin concentrations correspond with increased satiety and decreased energy intake at the subsequent meal (S. H. Holt, Brand Miller, & Petocz, 1996; Pal & Ellis, 2010; Verdich et al., 2001).

One of the ways that insulin controls food intake is through increasing sensitivity of the brain to satiety signaling hormones (Figlewicz, Stein, West, Porte, & Woods, 1986; Riedy, Chavez, Figlewicz, & Woods, 1995). Brain infusion of insulin further enhanced the ability of cholecystokinin (CCK) to reduce meal size as demonstrated in both rats and non-human primates

(Figlewicz et al., 1986; Riedy et al., 1995). This provides evidence that insulin works through multiple mechanisms and pathways to regulate energy intake and availability.

Insulinemia responses vary following meals of different protein sources (Floyd Jr, Fajans, Conn, Knopf, & Rull, 1966; S. Holt et al., 1997; Lang et al., 1999; Nilsson et al., 2004; Nuttall & Gannon, 1990; Sanchez & Hubbard, 1991). The insulin response from a meal containing whey (25% energy from protein) was greater than a meal of equal proportion protein coming from casein and soy protein (Veldhorst et al., 2009). This coincided with decreased subjective hunger following a whey protein meal compared to casein and soy. Further, digestion rate appears to influence insulin secretion. It was observed that casein protein meals delayed glucose and insulin responses by 1.5 hours following ingestion, as compared to soy protein meals (Lang et al., 1999). Differences between postprandial insulinemia following meals with egg white protein and wheat gluten protein may be contributing to increased satiety from the egg white protein meals.

Protein may be causing such distinct insulin responses because of peptides and/or amino acids released during digestion that is unique to each protein source. Milk had been found to produce some of the strongest insulin stimulatory effects of several protein sources that have been investigated (Hubbard et al., 1989; Liljeberg & Björck, 2001; Nilsson et al., 2004). Further investigation revealed that the strong insulinotropic response of milk protein may be due to the whey component. Insulin response to whey protein alone was even greater than the same amount of milk protein suggesting that the insulinotropic effects may be due to bioactive peptides or individual amino acids more pronounced in whey protein (Nilsson et al., 2004). Postprandial increases in plasma insulin levels have been demonstrated to correlate with changes in levels of plasma leucine, isoleucine, valine, phenylalanine, and arginine (Calbet & MacLean, 2002; van Loon et al., 2000). Dietary proteins vary in their capacity to simulate insulin release and evidence

suggests this may be due to insulinotropic plasma amino acids. It seems that protein stimulation of insulin release relies more on the products of protein digestion than the general increase in plasma amino acids.

Determining postprandial time-course changes in insulin is critical to understanding the involvement it could have on the differences in satiety between egg white and wheat gluten protein. Furthermore, it will be valuable to assess how the meal induced insulin changes correspond with fluctuations of amino acids measured in the previous study. The objective of this chapter is to assess postprandial changes in plasma insulin following consumption of meals containing either egg white or wheat gluten protein at two levels, 20% and 35% energy from protein. The results from this study will help identify the role (or lack thereof) that insulin has on the satiety responses following meals containing either protein source. It is hypothesized that egg white protein will stimulate insulin secretion to a greater extent than wheat gluten protein because of the greater ratio of insulinotropic plasma amino acids to other amino acids contained in egg white protein.

3.2 Methods

Animals. Male Sprague-Dawley rats (approximately 200 g; Charles River Laboratories) were singly housed in plexiglass cages (30 x 30 x 38 cm). The floors of the cages were fitted with steel wired bottoms resting approximately one inch above the cage floor in order to measure diet spillage. Animals were kept in a temperature-controlled room (26 ± 2 ˚C) and given free access to water. Upon arrival to our research facility, animals were trained to a reversed 12 h light:dark cycle (lights off at 800) and to a meal-feeding schedule. Acclimation to the diets and feeding schedule was designed to be a gradual process over 10 days. The process started with pelleted rodent chow (Harlan Teklabs, Madison, WI) being replaced with ground chow, food provided *ad*

libitum for 10 hours of the dark cycle and progressed into feeding test diets at restricted meal times. Food was removed one hour prior to the onset of the light cycle for an overnight fast. At one hour into the dark cycle (900 hr), animals were given 30 minutes to consume a meal, representing 20% average daily intake by weight (average of food intake on previous 3 days). Ground chow was available ad libitum between 1330 and 1900 hr. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois and are in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council).

Diet preparation. Diets were prepared to be comparable to AIN-93G (Reeves 1997). Separate mineral mixes were used to account for micronutrient differences in the egg white powder and wheat gluten powder. All diet components were purchased from Dyets, Inc. (Bethlehem, PA), with the exception of egg white powder and maltodextrin which were purchased from Harlan (Indianapolis, IN). The two high protein diets were approximately 45:35:20 (carbohydrate:protein:fat, as a % of calories) and the two normal protein diets 60:20:20 with protein provided by either egg white protein or wheat gluten protein.

Surgical procedure. Rats were acclimated for a one-week period prior to surgical procedure. Rats were anesthetized with isoflurane. Once anesthetized, the top of the head and the neck is shaved and the skin prepared with Povidone-Iodine 10%. A small (<3 cm) vertical incision is made in the neck to access a segment of the right jugular vein. A sterile Silastic catheter (0.025 in. ID x0.047 in. OD) filled with heparinized saline is inserted into the vein until reaching the right atrium, it is pulled back from the atrium for placement in the sinus of the superior vena cava. The catheter is secured to the vein with suture, tunneled under the skin and externalized through an incision made on the top of the head. The incision on the neck is closed with sterile

wound staples. Patency of the catheter was maintained by filling with a 90% glycerol solution (v/v) containing 500 U heparin/ml and capped with a sealed piece of Tygon tubing. Rats are then placed into a stereotaxic instrument (ASI Instruments, Warren, MI) and end of the catheter fixed in position with dental acrylic cement and anchored to the skull with four stainless steel screws. Following surgery, rats were monitored until they were fully recovered from the anesthesia and postsurgical analgesia provided by carprofen (5 mg/kg body weight s.c.) on the day of surgery and two days afterwards. Animals were evaluated daily for at least 7 days post-surgery.

Sample collection. Rats were given 5-7 days of ad libitum food access after surgery in order to regain lost weight and recover from the operation. Catheter patency was maintained by flushing catheters with heparinized 0.9% saline solution and filling them with 90% (v/v) glycerol solution containing 500 U/mL heparin. The flushing protocol was performed daily for 3 days following surgery and every other day afterwards. Only animals with body weights greater than on the day of surgery were used for sample collection. Once fully recovered, the feeding regimen resumed and the blood sampling protocol was initiated. On the day of sample, a 20 inch Tygon tube is connected to the externalized portion of the catheter at least one hour prior to sample collection. The other end of the tube is weighted on the outside of the cage so that collections can be made from unrestrained animals. A total of 6 samples were taken every 30 minutes, with the first sample taken 30 minutes before the test diet was given and the final sample taken 120 minutes (2 hours) after the test diet was given. Approximately 250 microliters of whole blood were collected at each time point and placed into EDTA coated microtainers. Each sample was briefly centrifuged for 10-15 seconds at 6000 rpm and 100 microliters of plasma were harvested. Blood volume was maintained by resuspending harvested red blood cells in a volume of 0.9% sterile saline solution equal to the amount of plasma removed at each time point. The resuspended red

blood cells were readministered to each animal through the jugular vein following collection of the next sample. Plasma samples were kept on wet ice through sample collection and kept at -80 degree Celsius until analysis.

Plasma Insulin analysis. Plasma insulin levels were determined using a rat insulin ELISA kit (EXRMI-13K, Billerica, MA). Samples were analyzed in duplicates. Acceptable coefficient of variance for plasma insulin was less than 15%.

Statistical Analysis. Data were analyzed using SAS v9.4 (SAS Institute Inc., Cary, NC). Levels of insulin were analyzed by repeated measures analysis with time-point (6 levels) as the within-subjects factor and Treatment (20EW, 20WG, 35EW, 35WG) as the between-subjects factor. We also collapsed the values across time-points and analyzed these values by 2-way ANOVA with concentration (20% or 35% protein), type (egg white or wheat gluten), and their interaction as factors. Tukey's least square difference (t-tests, with mean square error from ANOVA model) was used to evaluate pair-wise differences between means. Values were presented as means ± SEM. An alpha level of $P<0.05$ was considered statistically significant.

3.3 Results

Insulin levels increased following the meals, collapsed across treatment groups, as indicated by a significant effect of time in the repeated measures ANOVA ($F_{5,41}=6.62$, $P<0.0001$; Figure 3.1). Posthoc analysis indicated all time points following the meal (0-120 min), collapsed across treatment groups, were different from baseline (all p-values <0.05). A main effect of treatment was also observed ($F_{3,10}=8.37$, $P=0.004$), but no significant interaction between treatment and time point ($F_{15,42}=0.85$, $P=0.619$) was detected. Posthoc analysis of treatments collapsed across time showed plasma insulin concentrations were greater following 20EW than 35WG ($P=0.007$)

and 35EW (P=0.010). No other pairwise posthoc differences between treatments were significant.

In a separate analysis where insulin measurements were collapsed across all time points for each individual, a main effect of protein concentration was also observed ($F_{1,11}$=6.37, P=0.028; Figure 3.1). The 20% dose displayed approximately 41% higher insulin levels than 35% protein. Type of protein was not significant ($F_{1,11}$=1.28, P=0.281). Neither was the interaction between type and concentration.

3.4 Discussion

This study aimed to compare the insulinotropic effects of both protein source and protein concentration. The results demonstrated that rats provided 20% protein had greater overall increases in plasma insulin following meal consumption compared with rats fed meals containing 35% protein. Whereas, rats provided 20EW had a 2 hour postprandial insulin level averaging 8.56 ± 1.02 ng/mL, insulin responses in rats fed 35EW and 35WG were roughly half this value (4.81 ± 1.05 and 4.39 ± 1.15 ng/mL, respectively). Rats fed 20WG had an intermediate insulin response that was not significantly different from any of the other protein meals. These diets were designed so that differences in protein concentration (20% vs. 35%) comes at the expense of carbohydrates (60% vs. 45%, respectively). Meals of increased carbohydrate content are known to cause greater insulinemia (Foster-Powell, Holt, & Brand-Miller, 2002), suggesting that the increased insulin response following 20% protein meal ingestion may be more likely due to the increased carbohydrate component (60% carbohydrate) of the meal than the protein component. Taken together, the differences in insulin response observed when rats were fed varying protein diets demonstrate that protein source has limited effects on insulinemia, which favors the carbohydrate concentration of the meal being consumed.

The plasma insulin responses measured in this study did not correspond to treatment meal protein type. Within each protein concentration group, insulin response did not vary by source. Recall from Chapter 2 that each of the protein types produces different postprandial amino acid profiles and several studies suggest specific amino acids have insulinotropic properties. Nilsson and colleagues identified lysine, leucine, isoleucine, and valine to have the strongest positive correlation to insulin responses following protein meals in humans (Nilsson et al., 2004) and lysine, valine, and isoleucine were also found to increase following egg white protein meals (Chapter 2). Therefore, it is not clear why an effect of protein type on insulin was not detected. Calbet and MacLean also demonstrated that in addition to increased isoleucine and valine, leucine, phenylalanine, and arginine also corresponded closely to changes in insulin following pea, whey, and milk protein beverage ingestion (Calbet & MacLean, 2002). It is possible that much greater differences in plasma concentrations of valine, lysine, and isoleucine among protein test meals is required to stimulate a similar insulin response found in their study. Calbet and MacLean reported roughly 100 μmol/L differences of valine between protein meals, whereas, in our study, 35EW produced approximately 50 μmol/L greater valine levels than 35WG. Another study, found beverages supplemented with 0.4 g/kg/hour leucine, phenylalanine, and arginine to be the strongest stimulator of insulin secretion (van Loon et al., 2000). However, these three amino acids were not significantly different in our plasma analysis. It is possible that greater differences in specific plasma amino acids are required to induce an increase in insulin responses.

In addition to the data from this study being disparate to plasma amino acid response, these results also demonstrates differences in satiety observed in previous studies are unlikely to be due to postprandial insulin response. This is in opposition from other reports of postprandial

insulin levels having a negative relationship with post-meal satiety in lean human subject, with decreased ad libitum food intake following ingestion of foods and meals that produce greater insulin responses (S. H. Holt et al., 1996; Verdich et al., 2001). However, in these studies, changes in insulin levels were measured following carbohydrate manipulated meals. It is possible in these studies, that insulin secretion is a result of the ingested carbohydrate and is not involved in the mechanism causing protein-induced satiety. More similar to the results of the present study, several other studies have reported differences in protein-induced satiety among various protein types without significantly different postprandial insulin (Acheson et al., 2011; Diepvens, Häberer, & Westerterp-Plantenga, 2008; Hall, Millward, Long, & Morgan, 2003). Protein-induced satiety appears to occur through other mechanisms that is unlikely to be due to changes in levels of insulin.

Overall, results from this study did not support postprandial insulin responses to reflect plasma amino acid availability. Unlike postprandial plasma amino acid previously, no differences were detected between egg white and wheat gluten protein sources at either the 20% or 35% protein level. Insulin response appeared to be more of a reflection of the treatment meal's carbohydrate content. It is unlikely that previously observed increased satiety following ingestion of egg white protein meals compared to wheat gluten protein is related to changes in insulin levels.

3.5 Figure

Figure 3.1

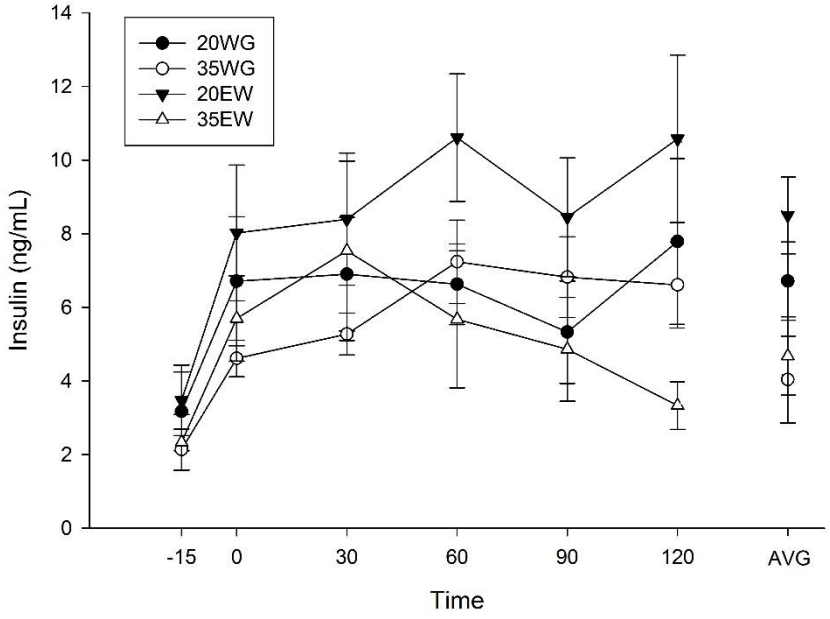

Figure 3.1. Effect of treatment meal on postprandial plasma insulin concentrations. Average concentration of plasma insulin ± SEM at each time-point, 15 minutes before the test meal, at the end of the test meal (time-point 0), and 30 min increments thereafter. Following the 120 min time-point, the least square mean (AVG) collapsed across all time-points is shown with the standard error from the ANOVA model. *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

3.6 References

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Chapter 4: Effect of protein quantity and source on acute and chronic feeding behavior and metabolism

4.1 Introduction

In addition to complement of amino acids and insulin signaling, differential energy expenditure following protein ingestion could contribute to the greater satiety induced from egg white as compared to wheat gluten. Meal composition can strongly influence diet-induced thermogenesis. Protein meals generally induce greater diet-induced thermogenesis than isocaloric meals of carbohydrates or fat (LeBlanc, Diamond, & Nadeau, 1991; Robinson et al., 1990). Because protein also induces the greatest satiety of the three macronutrients (Rolls, Hetherington, & Burley, 1988), it is thought that increases in diet-induced thermogenesis following protein intake could contribute to its satiety-enhancing effects. Furthermore, increasing protein content of meals induces greater diet-induced thermogenesis, corresponding to increased satiety ratings in high protein meals versus lower protein meals (Crovetti, Porrini, Santangelo, & Testolin, 1998). Therefore, this chapter will test the hypothesis that egg white protein induces a greater metabolic response than wheat gluten. If true, then the differential metabolic response constitutes another potential contributing factor to the differential satiety-inducing effects that were observed for these sources in previous work.

There are several proposed explanations for the relationship between increased energy expenditure and protein-induced satiety. The first has to do with the physiological response when the level of protein exceeds the capacity to store it. Protein and its amino acid constituents are unlike fats and carbohydrate, in that protein consumption in excess of an amount needed for protein synthesis cannot be stored (Halton & Hu, 2004). Excess amino acids have to be more quickly catabolized and eliminated than energy from carbohydrates and lipids and would

therefore increase energy expenditure to a greater degree. Additionally, metabolism of amino acids come at a greater energetic cost. Whereas, energy expenditure for ATP synthesis for glucose and lipid is 91.0 kJ/ATP and 96.3 kJ/ATP respectively, metabolism of amino acids costs more (Van Milgen, 2002). Energy expenditure for ATP synthesis for amino acids ranges from 99.2 kJ/ATP (for glutamate) through 153.2 kJ/ATP (for cysteine) (Van Milgen, 2002). Last, it is thought that the combined effect of increased oxygen consumption and body temperature from the increase in energy expenditure causes feelings of oxygen deprivation to induce satiety (Westerterp-Plantenga, Rolland, Wilson, & Westerterp, 1999). Overall, the hypothesis is that the energetically expensive catabolism of amino acids soon after ingestion of high protein meals contributes to perception of satiety.

Beyond high protein meals inducing greater energy expenditure, different protein sources can also vary in diet-induced thermogenic effects. 24-hour energy expenditure increased to a greater extent following a diet containing 29% energy from pork protein compared to the same amount of soy protein (Mikkelsen, Toubro, & Astrup, 2000). A separate study found a meal contain 50% energy from whey protein increased energy expenditure over the following 5.5 hours to a greater extent than meals with the same amount of protein from casein and soy protein (Acheson et al., 2011). Although each of the high protein meals caused increased fat oxidation when compared to the high carbohydrate meal, the meal containing whey protein increased fat oxidation to a greater extent than meals containing casein and soy protein. These results suggest that diet-induced thermogenesis is greater following a high protein meal from an animal protein source as compared to a plant protein source.

It is perhaps the difference in amino acid composition of each protein source that contributes to variation in energy expenditure. The variation in carbon side chains of amino acids

means that metabolism of each of the twenty amino acids is unique to the stoichiometry. Glutamate makes up a significant proportion of the composition of wheat gluten protein at 33.1 g per 100 grams of protein (Watson, 1996), and also happens take the least amount of energy of any of the amino acids to catabolize (Van Milgen, 2002). The large proportion of glutamate comes at the cost of other amino acids. Compared to wheat gluten protein, egg white protein contains higher amounts of amino acids that are energetically more expensive to catabolize, such as arginine, serine, tyrosine, and phenylalanine (Watson, 1996). Difference in amino acid composition between egg white and wheat gluten protein could contribute to increased energy expenditure following ingestion of egg white protein to drive satiety.

Given that acute responses to high protein diets result in increased energy expenditure, high protein diets may aid in weight management by inducing favorable metabolic changes when consumed over an extended period. Rats maintained on a high protein diet (50% energy from protein) for 21 days had decreased food intake and reduced fat mass with increased ratio of fat to lean mass when compared to rats fed a diet of 14% protein (Jean et al., 2001). Additionally, these rats had increased liver enzymes involved in the transport and catabolism of amino acids. Chronic consumption of high protein diets also increases expression of tissue specific uncoupling proteins 1 and 2 which are thought to be involved in substrate oxidation and thermogenesis (Petzke, Friedrich, Metges, & Klaus, 2005). It should be noted that 50% protein is much greater than normal rodent diet (10-20% energy from protein) and even human diets, which average 14% energy from protein and can increase to 25-35% energy from protein when mindfully adhering to a high protein diet (Fulgoni, 2008; Heather J Leidy et al., 2015). It is necessary to assess long term consumption of increased protein in a manner that is realistic for translating to human behavior and physiology. While it does appear that long term consumption of high

protein diet has positive benefits to body composition, there is currently minimal understanding of the underlying metabolic changes associated with increased protein intake.

It is therefore, the objective of this study to first assess protein source and quantity on acute responses of feeding behavior and metabolism. Secondly, this study was designed to assess long-term changes to feeding patterns, body weight, body composition and metabolism in response to manipulating the protein component at the first meal of the day. It is hypothesized that a meal of higher levels of egg white protein would increase protein metabolism and energy expenditure to correspond to increased satiety. Over an extended period, it is expected that regular ingestion of increased egg white protein have favorable metabolic outcomes.

4.2 Methods

Animals. Adult Sprague-Dawley rats (4 months; Envigo, Indianapolis, IN) were singly housed in a temperature-controlled room (26 ± 2 ˚C) with a 12 hr reverse light cycle (light on at 2000 hr). Rats were provided ad libitum access to water. Upon arrival to our research facility, animals were allowed one week to acclimate prior to being trained to a meal-feeding schedule. Training to the diets and feeding schedule was designed to be a gradual process over 8 days. The process started with pelleted rodent chow (Harlan Teklabs, Madison, WI) being replaced with powdered AIN93M (Research Diets, New Brunswick, NJ), food provided ad libitum for 10 hours of the dark cycle and progressed into feeding test diets at restricted meal times. Food was removed one hour prior to the onset of the light cycle for an overnight fast. At one hour into the dark cycle (900 hr), animals were given 30 minutes to consume a meal, representing 10% average daily intake by weight (average of food intake on previous 3 days). Powdered AIN93M was available ad libitum 5.5 hrs between 1330 and 1900 hr. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois and are in

accordance with the Guide for Care and Use of Laboratory Animals (National Research Council).

Diet preparation. Testing diets were prepared to be comparable to AIN-93G (Reeves 1997). Separate mineral mixes were used to account for micronutrient differences in the egg white powder and wheat gluten powder. All diet components were purchased from Dyets, Inc. (Bethlehem, PA), with the exception of egg white powder and maltodextrin which were purchased from Harlan (Indianapolis, IN). The two higher protein diets were approximately 45:35:20 (carbohydrate:protein:fat, as a % of calories) with protein provided by either egg white protein or wheat gluten protein. The two normal protein diets were approximately 60:20:20 (carbohydrate:protein:fat, as a % of calories) with protein provided by either egg white protein or wheat gluten protein.

Indirect calorimetry. Comprehensive Laboratory Animal Monitoring Systems (CLAMS) (Oxymax; Columbus Instruments International, Columbus, OH, USA) allowed continuous quantification of the duration and amount of food intake, as well as measured carbon dioxide production and oxygen consumption at 13 minutes intervals for 23 hr periods. Respiratory exchange ratio (RER) was calculated as V_{CO2} production / V_{O2} consumption. Heat production was calculated using the following equation:

$$Heat = 3.815 + 1.232 * V_{CO_2}$$

Food intake during the test meal was closely monitored so that feeders were manually opened at one hour after the start of the dark phase and were closed as soon as the allotted 10% of average daily intake was consumed or the 30 minutes meal period was over, which ever event came first. Feeders were removed following the test meal and replaced within 30 minutes with powdered

AIN93M for ad libitum consumption for 5.5 hrs between 1330 and 1900 hr unless noted otherwise. Meals were defined as being the total of all bouts in which consumption was greater than 0.03 grams and with the time between each bout being no longer than 10 minutes. Rats were allowed two days to acclimate to the cages before data was collected on subsequent days.

Experiment 1: Acute response study. This study was completed over two cohorts with male (n=12) and female (n=12) rats. Following acclimation to being housed in CLAMS, feeding behavior and metabolic responses were assessed following ingestion of test meal on alternating days until each rats received each of the four test diets. Test meal was provided at the first meal of the day based on evidence that protein consumption at breakfast has the largest impact on satiety (H. J. Leidy, Bossingham, Mattes, & Campbell, 2009). Treatment diets at the test meal were balanced across data collection days and sex. Feeders were reopened for rats to access powdered AIN93M at 1000 hr on testing days to measure subsequent food intake. Rats were given a day between testing data collection days in order to avoid learned anticipation of food access following testing meal and to provide a washout period. On these intermediate days, rats were provided powdered AIN93M during the first meal period and ad libitum food intake was restricted to be between 1330 and 1900 hr. Data from days in which rats did not complete provided meals were removed from analysis. This occurred in the removal of eight data sets in the 35EW treatment group (3 males, 5 females) and two data sets from the 20EW (both females).

Experiment 2: Chronic diet intervention study. This diet intervention study took place over 30 days and completed in two cohorts. Male rats were pseudo-randomly assigned to the four diet treatment groups (n=6/treatment), balanced for baseline body weight. The same treatment diets were provided at the first meal of the day over the 30-day intervention with food intake and body weight measurements taken daily. Indirect calorimetry data were collected for the initial and

final three days of the 30-day intervention. Data from three days following acclimation to being housed in CLAMS were averaged for each animal prior to statistical analysis. Body composition (lean and fat mass) was evaluated by EchoMRI (EchoMRI Medical Systems, Houston, TX) at baseline (Day -2), middle (Day 15), and at the end of the diet intervention study (Day 31).

Statistical Analysis. Data were analyzed using SAS v9.4 (SAS Institute Inc., Cary, NC). Food intake was analyzed by three-way analysis of variance (ANOVA) with main effects of sex (male or female), protein source (wheat gluten or egg white), and protein level (20% or 35%). Changes in body weight and body composition were analyzed by repeated measures analysis with date as the within-subject factor and treatment (20EW, 20WG, 35EW, 35WG) as the between-subject factor. Changes in RER and heat were analyzed by repeated measures analysis with time as the within-subject factor and treatment (20EW, 20WG, 35EW, 35WG) as the between-subject factor. Posthoc tests analyzed individual time points using 2-way ANOVA with protein level and protein source as factors. Tukey's least square difference (t-tests, with mean square error from ANOVA model) were used to evaluate pair-wise differences between means. Values were presented as means ± SEM. An alpha level of $P<0.05$ was considered statistically significant.

4.3 Results

Experiment 1: Acute response study

Subsequent meal size differed between breakfast treatment groups depending on the protein source ($F_{1,78}=9.25$, $P<0.005$) (Figure 4.1a). Rats provided meals containing egg white protein had decreased food intake at the subsequent meal compared to wheat gluten fed rats ($P=0.003$). Whereas egg white protein fed rats had a subsequent meal size of 2.810±0.257 grams, wheat gluten protein fed rats consumed 3.847±0.224 grams. No effect of protein level on

subsequent meal size was detected. Neither protein level nor source affected the food intake for the duration of the day (Figure 4.1b).

Sex differences with regards to food intake were also observed. Male rats had a significantly larger subsequent meal ($F_{1,78}$=43.17, P<0.001) compared to female rats, 4.449±0.233 grams and 2.209±0.249 grams respectively (Figure 4.1a). Sex differences were also observed in food intake for the remainder of the day ($F_{1,78}$=43.02, P<0.001) with males expectedly consuming more than females, 17.410±0.350 grams and 14.055±0.373 grams respectively (Figure 4.1b). No interactions between sex and level or source of protein were observed.

The respiratory exchange ratio (RER) increased following the test meal and stayed elevated until that start of the light cycle at 1000 (Figure 4.2a). The change in RER was indicated by a significant main effect of time ($F_{23,506}$=624.20, P<0.001). Overall, the RER for next 20 hours following the 20WG treatment meal was greater than the other treatment meals ($F_{1,78}$=43.17, P<0.001). This was indicated by posthoc analysis showing RER following 20WG to be greater than 20EW (P<0.001), 35EW (P<0.001), and 35WG (P<0.001). No other pair-wise differences were detected. An interaction of time and treatment was also detected ($F_{69,1242}$=1.32, P<0.050), which was driven by decreased RER in the 35EW group during the first three hours after treatment meal consumption.

No significant differences between treatment groups were detected in energy expenditure (Figure 4.2b). Energy expenditure was observed to be more elevated during the dark cycle compared to the light cycle as indicated by a significant main effect of time ($F_{23,509}$=496.77,

P<0.001). Males had greater energy expenditure than females ($F_{1,22}$=54.45, P<0.001). No interactions were significant.

Experiment 2: Chronic diet intervention study

Rats in all four treatment groups displayed increased body weight over the course of the study ($F_{2,40}$=153.45, P<0.001) (Figure 4.3a). No significant differences were observed between treatment groups. Fat mass ($F_{2,40}$=114.68, P<0.001) and lean mass ($F_{2,40}$=55.18, P<0.001) increased over the 30-day intervention period in all four treatment groups, however changes in fat and lean mass did not differ between treatment groups (Figure 4.3b and 4.3c). Neither did the ratio of fat to lean mass (Figure 4.3d). Daily food intake was not significantly different between treatment groups (data not shown).

RER changed over the course of the day as reflected by a significant effect of time ($F_{21,420}$=143.45, P<0.001) (Figure 4.4a and 4.4b). During both the initial and final days of the intervention, RER increased soon after ingestion of the breakfast meal and decreased over the following four hours during which access to food hopper was restricted. This increase in RER was also apparent during the ad libitum time period of the feeding regimen. Overall RER decreased from the initial to final days of being of the diet regimen as indicated by a significant difference between baseline and final day ($F_{1,20}$=167.03, P<0.001).

An interaction between time and treatment was observed for RER during the initial ($F_{21,420}$=453.15, P<0.001) and final ($F_{21,420}$=650.02, P<0.001) days of being on the diet regimen (Figure 4.4a and 4.4b). Results indicated the treatment differences were apparent for the first four hours following the treatment meal, hence separate analyses were conducted for this each of these time points. Results indicated a significant effect (P<0.05) of protein level for the first two

hours during initial day and three hours on the last days. A significant effect ($P<0.05$) of protein type was detected during the first two hours during both initial and final days.

Overall energy expenditure decreased by 2% over 30-day intervention period as indicated by comparing total energy expenditure at baseline versus the final day ($F_{1,20}=8.25$, $P=0.009$) (Figure 4.5a and 4.5b). Energy expenditure significantly changed across the day, with higher expenditure during the night than daytime ($F_{1,20}=2145.89$, $P<0.001$). No significant differences of energy expenditure were detected between diet treatment groups (Figure 4.5a and 4.5b).

4.4 Discussion

The results of this study demonstrate that the protein source provided at the first meal of the day has an effect on the acute measures collected herein (food intake, RER), but the effect dissipates with chronic intake. We found that ingestion of test meals containing egg white protein caused smaller subsequent meal size when feeders were reopened 30 minutes later, an indication of increased satiety. Additionally, ingestion of the test meal containing a greater concentration of egg white protein caused lower respiratory exchange ratio during the first several hours. Decreased food intake following the test meal did not influence overall food intake or energy expenditure through the remainder of the day. In the second experiment, animals were kept on the feeding regimen and given treatment diets as the first meal for an extended period of 30 days to assess the effects of chronic intake. Contrary to the hypothesis, feeding a protein treatment at the first meal of the day over the course of 30 days did not have an effect on body weight, body composition, energy intake, or energy expenditure. Together, these data suggests that the effects of protein source as a component of a meal is short lived and does not show extended effects in the measurements made in this study.

This study provided further confirmation that egg white protein is more satiating than wheat gluten protein. In a previous study, it was found that rats given a 35% egg white protein meal had greater satiety than when provided a 35% wheat gluten protein meal (Du, Markus, Fecych, Rhodes, & Lee Beverly, 2017). In experiment 1, the acute response study, we found that at both 20% and 35% protein, rats given meals containing egg white protein ate less at the subsequent meal when feeders were reopened 30 minutes later compared to rats given meals containing wheat gluten protein. However, when feeder were instead reopened 4 hours later, as in experiment 2, differences in meal size among treatment groups were not significantly different suggesting the satiety effect is limited to the first several hours following test meal ingestion. Taken together with my earlier work, these data provide clear and repeatable evidence to support egg white protein meals inducing greater satiety than meals containing wheat gluten protein.

In addition to comparing the effects of protein source on food intake and metabolism, this study also compared the effects of protein at two different concentrations, 20% and 35%. Our results, which show no difference in satiety following meals of 20% and 35% protein, is in opposition to other studies demonstrating higher protein meals increase satiety (Heather J Leidy et al., 2015). Nevertheless, it is important to note that the difference in protein concentration between low and high protein groups in several of these studies are much greater than the amounts used in the present study. For example, one group compared the satiety effects of treatments containing 14% versus 50% protein (Bensaïd et al., 2003). A possible reason for the lack of difference in food intake following ingestion of 20% and 35% protein meals in the present study could be that both levels of protein meet minimum threshold to trigger protein-induced satiety (Paddon-Jones & Leidy, 2014). Differences in satiety measurements are more likely to be detected when low protein treatment groups fall below 15% protein and high protein

treatment groups contain greater than 25% protein (Heather J Leidy et al., 2015). Because the level of protein used in this study are both greater than adequate, difference is satiety may be more difficult to detect.

Experiment 1 also explored sex differences in food intake and metabolism following ingestion of protein treatment meals. As is expected, females consumed roughly 3 grams less food compared to their male counterpart and this was reflected in a 15-20% decrease in heat production (Council, 1995). And although females received a smaller first meal, as it is calculated as a proportion of total food intake, subsequent meal sizes were also smaller. However, the effects of the dietary treatments were consistent in both males and females. These results confirm that the decreased food intake and satiety from ingesting egg white protein meals extend to both male and female rats.

RER, a ratio of carbon dioxide production and oxygen utilization, provides an indication of energy source being metabolized. While RER collapsed over the course of the day did not differ among treatment groups, RER was found to be lower in 35EW during the first several hours following treatment meal ingestion. In all four treatment groups, RER immediately increases following ingestion of the meal following an overnight fast, indicating that the food intake caused metabolism to move away from lipid utilization when an external source of energy was provided. In experiment 1, 35EW caused significantly lower RER compared to 35WG at one hour post-meal ingestion. The lower RER of roughly 0.92 observed in 35EW at the 11am timepoint suggests that carbohydrates are predominately being oxidized, but protein oxidation is likely also occurring. Interestingly, this difference was detected even though animals in all treatment groups had *ad libitum* access to a control diet. In experiment 2, RER decreased following meal consumption due to food restriction for the next four hours. After 30 days of

adaptation, differences of RER between protein sources became more notable, suggesting that perhaps metabolism of protein sources are more efficient. As was expected, higher protein treatments caused lower RER for the several hours following meal ingestion at a level that indicates increased protein oxidation (Stepien et al., 2010). Differences in RER were not detected in 20% protein treatment groups, suggesting that the effect of protein source on RER is limited to meal compositions in which protein makes up greater than 20% energy. The decreased overall RER of animals across all four treatment groups by the end of the 30 day trial is suggestive of adaptation and increased efficiency of utilizing dietary protein for fuel. This study supports prior evidence of metabolic adaption to increased dietary protein (Jean et al., 2001; Petzke et al., 2005). Overall, RER appears influenced by protein source at higher concentrations of protein for the first 4 hours following meal ingestion, which becomes more pronounced with chronic ingestion.

In addition to measuring RER to uncover the metabolic effects of the treatment diets, energy expenditure was also assessed through the calculation of heat production. These results show both subsequent and all day energy expenditures to be similar amongst treatment groups following both acute and chronic feeding. While there are studies that found differences in energy expenditure following ingestion of meals containing different protein sources (Acheson et al., 2011; Alfenas, Bressan, & Paiva, 2010), other studies have also similarly found no differences between whey and casein (Bendtsen et al., 2014; Lorenzen, Frederiksen, Hoppe, Hvid, & Astrup, 2012). It is possible that protein sources may only be causing differential energy expenditure at much higher proportions of dietary protein intake as these studies provided subjects with 50-60% protein (Acheson et al., 2011; Alfenas et al., 2010). Furthermore, these results differ from clinical studies which have shown that high protein diets when compared to

low protein control diet increased subsequent and 24-hour energy expenditure (Halton & Hu, 2004). However, even from the studies which have found positive results, differences in energy expenditure between treatment groups are modest (Halton & Hu, 2004). Additionally, our results showing differences in RER, but not in energy expenditure is supported by the findings of Stepien and colleague (Stepien et al., 2010). In their study, rats adapted to a high protein diet (55% protein) did not change basal, total, or diet-induced energy expenditure. They found slightly increased lipid oxidation complemented by a slight decreased carbohydrate oxidation during the earlier period of adaptation, but both measures were restored by the end of the 14 day study. Together, these results demonstrates that it is unlikely for any changes in energy expenditure, both during the subsequent period and all day, to play a role in protein-induced satiety from ingestion of egg white protein meals.

Overall, this study provided further evidence demonstrating that meals containing egg white protein induced greater satiety than wheat gluten protein. Although protein source has significant short term implication for satiety and metabolism, manipulation of the protein component of a single meal of the day has minimal long term effects on body weight and composition. Given the short-lived effects of a high protein meal, any effects on body weight and composition is likely to require continuous consumption of increased protein meals throughout the day.

4.5 Figures

Figure 4.1

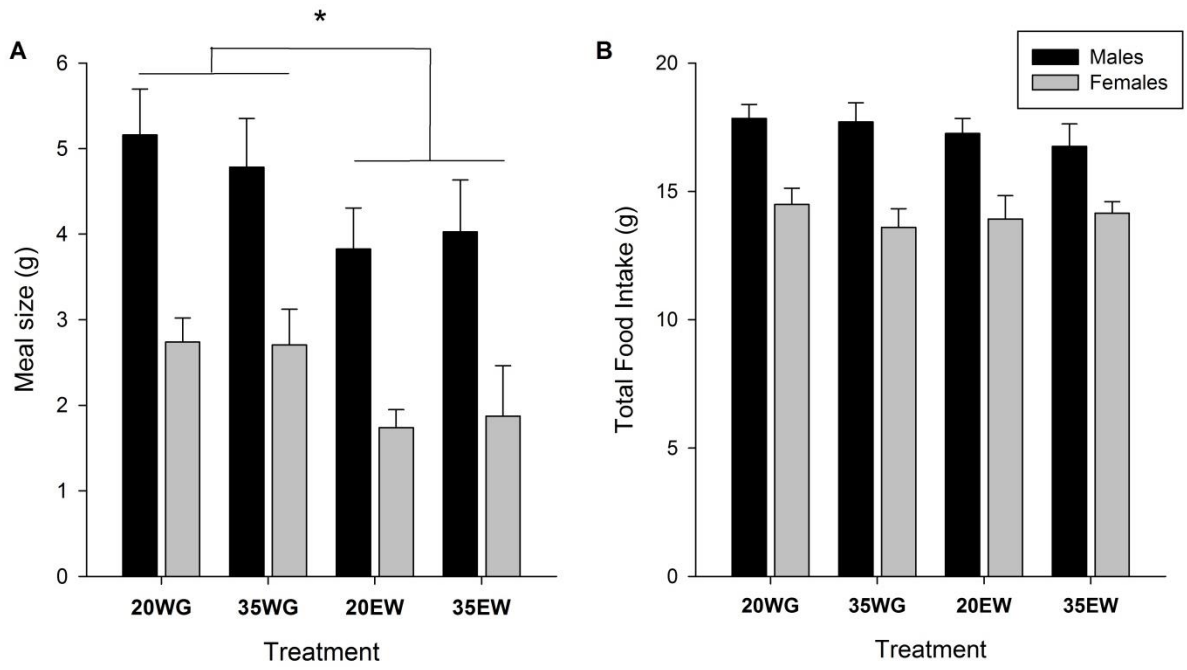

Figure 4.1. Food intake following treatment meals (20WG, 35WG, 20EW, or 35EW) (Experiment 1). (A) Effect of treatment meal on total amount consumed in subsequent meal. (B) Effect of treatment meal on total amount consumed throughout the remainder of the day. Bars represent mean ± SEM. * indicates a significant main effect of treatment ($P<0.05$). *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

Figure 4.2

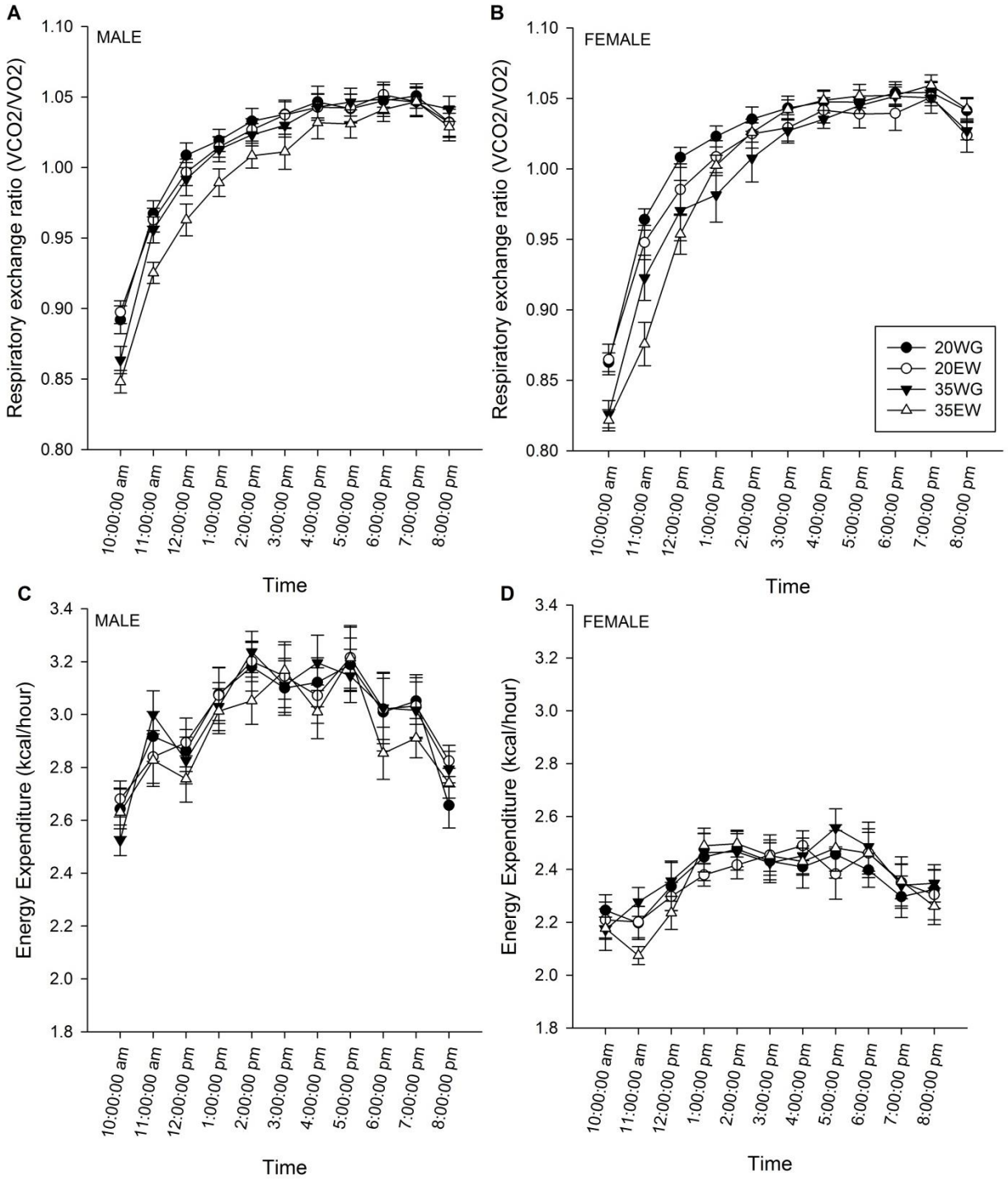

Figure 4.2. Metabolic responses of treatment meals (20WG, 35WG, 20EW, or 35EW) for the following 11 hours of the dark cycle. (A) Effect of treatment meal on respiratory exchange ratio (RER; calculated as V_{CO2} production / V_{O2} consumption) in male rats. (B) Same as A in female rats. (C) Effect of treatment meal on energy expenditure (kcal/hour). Bars represent mean ± SEM. (D) Same as C in female rats. *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

Figure 4.3

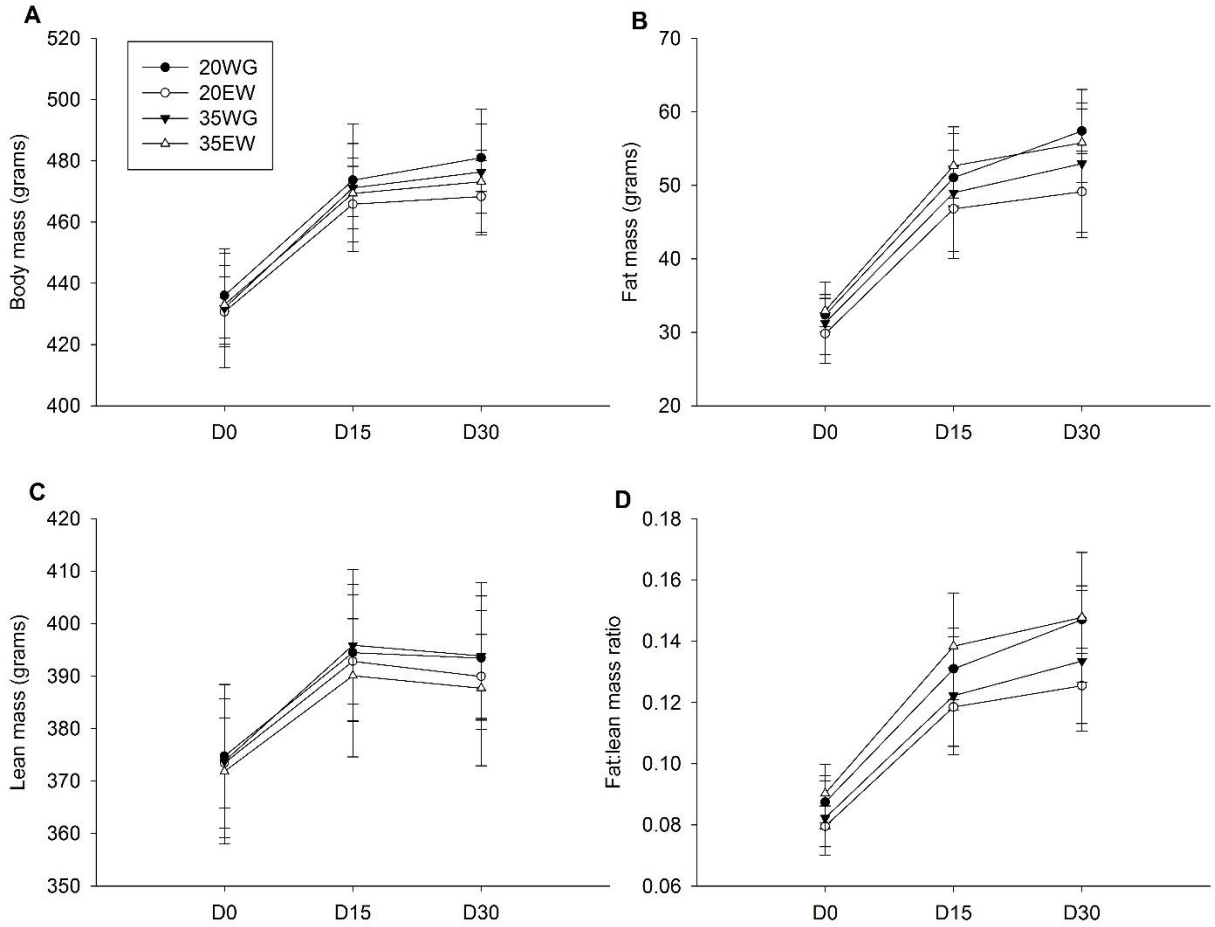

Figure 4.3. Effect of chronic treatment meal regimen (20WG, 35WG, 20EW, or 35EW) on body composition measurements (Experiment 2). (A) Effect of treatment meal regimen on average body mass ± SEM from baseline (D0), 15 days (D15), and at the end of the 30 day regimen (D30). (B) Same as A for fat mass. (C) Same as A for lean mass. (D) Same as A for the fat to lean mass ratio. *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

Figure 4.4

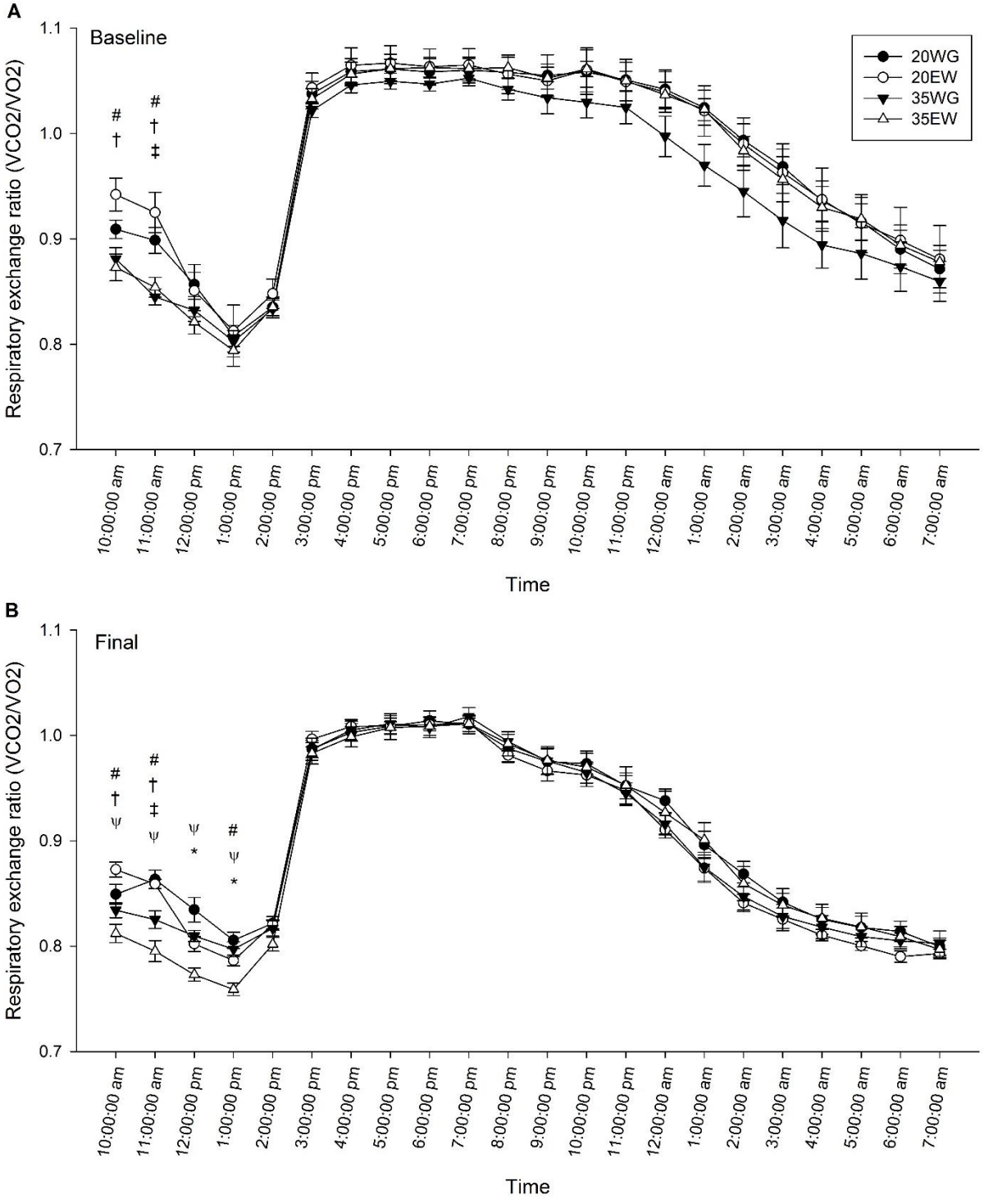
A
Baseline
20WG
20EW
35WG
35EW
Respiratory exchange ratio (VCO2/VO2)
1.1
1.0
0.9
0.8
0.7
10:00:00 am
11:00:00 am
12:00:00 pm
1:00:00 pm
2:00:00 pm
3:00:00 pm
4:00:00 pm
5:00:00 pm
6:00:00 pm
7:00:00 pm
8:00:00 pm
9:00:00 pm
10:00:00 pm
11:00:00 pm
12:00:00 am
1:00:00 am
2:00:00 am
3:00:00 am
4:00:00 am
5:00:00 am
6:00:00 am
7:00:00 am
Time
B
Final
Respiratory exchange ratio (VCO2/VO2)
Time

Figure 4.4. (continued). Effect of 30 day treatment meal regimen (20WG, 35WG, 20EW, or 35EW) on respiratory exchange ratio (RER; calculated as V_{CO2} production / V_{O2} consumption). (A) Hourly RER during initial days of treatment meal intervention. (B) Hourly RER during final days of treatment meal intervention. Bars represent mean ± SEM. Symbols indicates a significant different between treatment groups by posthoc t-tests ($P<0.05$). *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

Figure 4.5

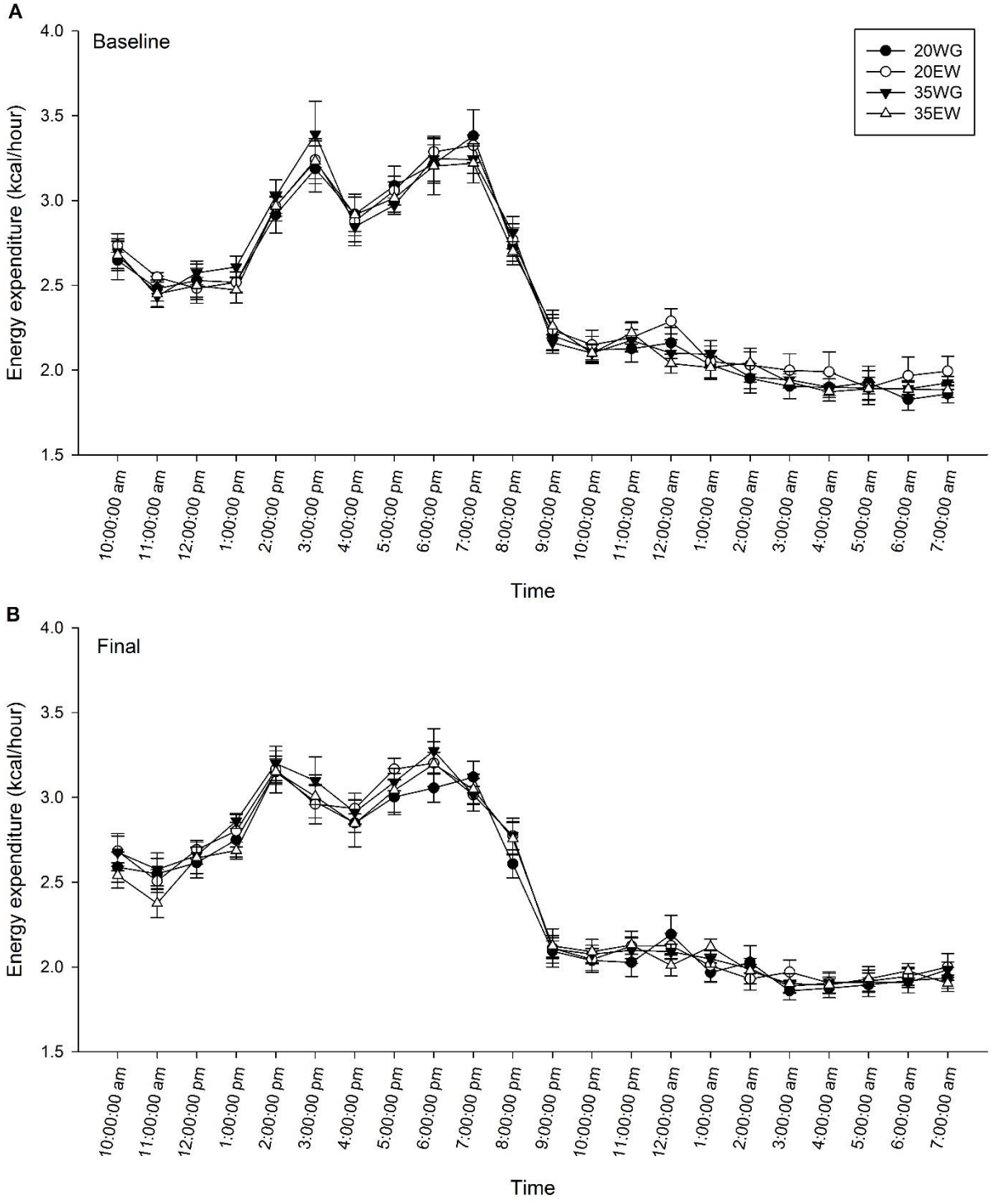

Figure 4.5. Effect of 30 day treatment meal regimen (20WG, 35WG, 20EW, or 35EW) on energy expenditure. (A) Hourly energy expenditure during initial days of treatment meal intervention. (B) Hourly energy expenditure during final days of treatment meal intervention. Bars represent mean ± SEM. *20WG, 20% Wheat gluten diet; 35WG, 35% Wheat gluten diet; 20EW, 20% Egg white diet; 35EW, 35% Egg white diet.*

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Chapter 5: Conclusion and Future Directions

Results from this dissertation establish that meals containing egg white protein induce greater satiety than wheat gluten protein, corresponding with increased postprandial plasma amino acids and lowered respiratory exchange ratio. These results add valuable new evidence to a large body of literature that currently favors high protein diets as a weight loss strategy and for treating obesity. The crucial new piece of information from my dissertation is that the source of protein matters to a large degree when evaluating the satiety enhancing effects of a high-protein diet composed of wheat gluten or egg white. Therefore, an important implication of the work from this dissertation is the importance of considering source of protein when designing high-protein diets for their satiety-inducing effects.

Although dietary source of protein had significant short-term implications for satiety and metabolism, manipulation of the protein component of a single meal of the day had minimal long-term effects on body weight and composition. Meals containing egg white protein caused subsequent decreased food intake and in the case of 35% egg white protein treatment lowered respiratory exchange ratios (Figure 4.1a & 4.2a). However, these feeding and metabolic responses were short lived lasting for only up to 4 hours following meal ingestion (Figure 4.4). Protein treatment groups did not differ in 24-hour food intake or 24-hour energy expenditure (Figure 4.5). Neither were there differences detected in body weight and body composition when the meal regimen was maintained for 30 days (Figure 4.3). Given the short-lived effects of a high protein meal, any effect on body weight and composition will be more likely to require continuous consumption of increased protein meals throughout the day. Future studies should consider testing chronic feeding of these different protein sources throughout the day over

extended periods to see whether it may affect overall food intake, body weight and body composition.

In addition, to establishing short term differential satiety-inducing effects from egg white versus wheat gluten, this dissertation also explored a few different potential mechanisms for the enhanced satiety-inducing effect of egg white protein. Meals containing egg white protein induced a different postprandial plasma amino acid profile than wheat gluten in ways that may partially explain the satiety effect (Figure 2.1 and 2.2). Egg white protein increased lysine, isoleucine, valine, and tryptophan more than wheat gluten protein, amino acids that have been identified in other studies to have anorexigenic effects (Ayaso, Ghattas, Abiad, & Obeid, 2014; Hall, Millward, Long, & Morgan, 2003; Jordi et al., 2013; Veldhorst et al., 2009). Taken together, these results suggest that the satiety enhancing effects of egg white protein are correlated with increased overall plasma amino acid levels, as well as several specific amino acids. To establish causation, future studies should explore the extent at which infusion of these amino acids at physiologically relevant levels directly into circulation could decrease food intake and thus contribute to satiety signaling.

Increased insulin signaling after egg white protein versus wheat gluten protein meals had been hypothesized to be involved in the differential satiety inducing effects of these protein sources. However, this does not appear to be the case as no differences in insulin levels were detected between egg white and wheat gluten protein sources at either the 20% or 35% protein levels following the meals (Figure 3.1). Instead, the insulin response reflected the treatment meal's carbohydrate content, with greater insulin after 20% than 35% protein meals due to increased carbohydrates in the 20% protein diets.

While insulin levels were not affected by the protein source ingested in this dissertation, future studies should investigate other satiety hormones that could be differentially released in response to egg white protein versus wheat gluten meals and contribute to the differential satiety-inducing effects of these protein sources. Other hormones that should be investigated include cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and peptide YY (PYY). Evidence suggests that amino acids and oligopeptides released during dietary protein digestion causes release of these satiety hormones to transmit information about energy intake to the central nervous system (Batterham et al., 2006; Moran, 2009; Raybould et al., 2006). Additionally, ghrelin release from the stomach is inhibited in the presence of amino acids in the gastrointestinal tract (Overduin, Frayo, Grill, Kaplan, & Cummings, 2005), and therefore decreased ghrelin is thought to also be involved in satiety signaling. One obstacle to measuring these hormones, though, is that they are highly sensitive to degradation and require large sample sizes for accurate analysis. Although the use of the MILLIPLEX Multiplex assay (Millipore, Billerica, MA) had been promising for its ability to measure many of these hormones from a single small sample of rodent plasma, in preliminary studies I found its ability to detect the hormones of interest were limited. Analysis of CCK is especially limited by its requirement for large sample sizes and a very specific antibody that does not cross-detect gastrin, another hormone found in much greater concentration with a close homology to CCK (Rehfeld, 1998). CCK, GLP-1, PYY, and ghrelin remain high yield and of great interest for their potential to differ following ingestion of different protein sources and thereby impact satiety outcomes.

There are several other limitation in this dissertation that needs to be considered in planning future research. First, this study uses two concentrations of protein (20% and 35% protein) that are greater than the standard amount of protein (12-18% protein) in the average rodent and

human diet. The concentrations tested in this dissertation were selected to match the 90th percentile of protein intake (20% protein) and a greater level more typically recommended in high protein diets (35% protein). While the concentrations of protein used in this study are within range of the amount of protein consumed in high protein diets, it is possible that these higher concentrations of protein surpass minimum threshold levels required to trigger protein-induced satiety (Paddon-Jones & Leidy, 2014). This may be why food intake was comparable following ingestion of 20% and 35% protein (Figure 4.1a). It may be of interest to evaluate food intake and other related outcome measurements following meals of egg white and wheat gluten protein at levels that are more typical of the average human and rodent diets (about 15% protein) or that meet minimum protein requirements (10% protein) (Fulgoni, 2008; Micronutrients, 2005). Secondly, the method of amino acid analysis used was limited to the measurement of ten out of the twenty amino acids. While most of the more important essential amino acids were included in the analysis, future studies would benefit from a complete analysis of all twenty amino acids. This may reveal additional amino acids that differ among protein treatment groups and tell a more complete story of how the amino acid levels correlated with the satiety response. Hence, future studies should examine satiety effects of meals that include lower percentages of protein, and include analysis of all amino acids.

Increased diet-induced thermogenesis has previously been suggested to be involved in protein-induced satiety (Acheson et al., 2011; Westerterp-Plantenga, Rolland, Wilson, & Westerterp, 1999), and was therefore predicted to be involved in the difference in satiety following egg white and wheat gluten protein meals. However, results from this study showed energy expenditure following ingestion of protein treatment meals to be similar. Nonetheless, the test meal containing a greater concentration of egg white protein caused lower respiratory

exchange ratio during the first several hours, an indication of increased protein metabolism in the 35% egg white protein treatment group. These results suggest increased efficiency of the metabolism of egg white protein when provided at higher concentrations.

Altogether, results of my dissertation illustrate the importance of considering protein source when designing high-protein diets to control appetite. This is especially important considering another one of my previous study showing 35% wheat gluten protein meals to have reduced satiety compared to 20% protein control meal (Du, Markus, Fecych, Rhodes, & Lee Beverly, 2017). The current protein literature suggests that whey and pea proteins have potent satiety inducing effects (Abou-Samra, Keersmaekers, Brienza, Mukherjee, & Macé, 2011; Diepvens, Häberer, & Westerterp-Plantenga, 2008; Veldhorst et al., 2009). My work adds egg white protein to that list, shows that wheat gluten has limited effects on satiety, and therefore should be avoided when formulating high-protein diets for weight management. It would be of interest to study how pea and whey compares to egg white protein in inducing satiety, and the mechanisms that underlie the different satiety effects from these protein sources. Given the obesity epidemic, and the large number of subjects in the United States and around the world that are incorporating a high-protein diet into their life in order to manage weight gain, it is crucial that we establish the fundamental knowledge about the satiety inducing effects of specific protein sources and resulting post-prandial amino acid profiles. Such knowledge will allow us to make intelligent evidence-based decisions about our diet and health moving forward.

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